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Cytochrome P450s are a common type of the polyketide tailoring enzymes that are responsible for the oxidation of polyketides. PksS, a likely tailoring enzyme, is found within the gene cluster *pksX*, from *Bacillus subtilis*. The gene cluster was found to synthesize bacillaene, a polyketide with antibiotic activity. Sequence homology has classified *pksS* as a cytochrome P450. We have hypothesized that PksS is responsible for the hydroxylation of bacillaene, dihydrobacillaene, or both. After several attempts to clone, overexpress, and purify *pksS*, we determined a sequence error within the published sequence. Following the determination of the correct sequence, the next in-frame stop codon was located. Using this newly identified sequence, PksS was overexpressed successfully and assayed using a spectrophotometric cytochrome P450 assay that detects a carbon-monoxide complex with the ferrous form of the P450. Spectra obtained definitively showed that PksS is a cytochrome P450.

Several steps have been taken to characterize the role PksS serves in the biosynthesis of polyketides by *B. subtilis*. High-pressure liquid chromatography, coupled to mass spectrometry has preliminarily shown that the substrate for PksS is dihydrobacillaene. Other attempts to construct mutations of *pksS* have been performed, but experiments with the mutant strains did not yield conclusive information.

CHARACTERIZATION OF *PKSS* IN *BACILLUS SUBTILIS*

by

Stephanie Anne Antolak

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Approved by

Committee Chair

To my parents:

Your constant love, support, and guidance
continues to inspire me

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

Committee Chair _____

Committee Members _____

Date of Acceptance by Committee

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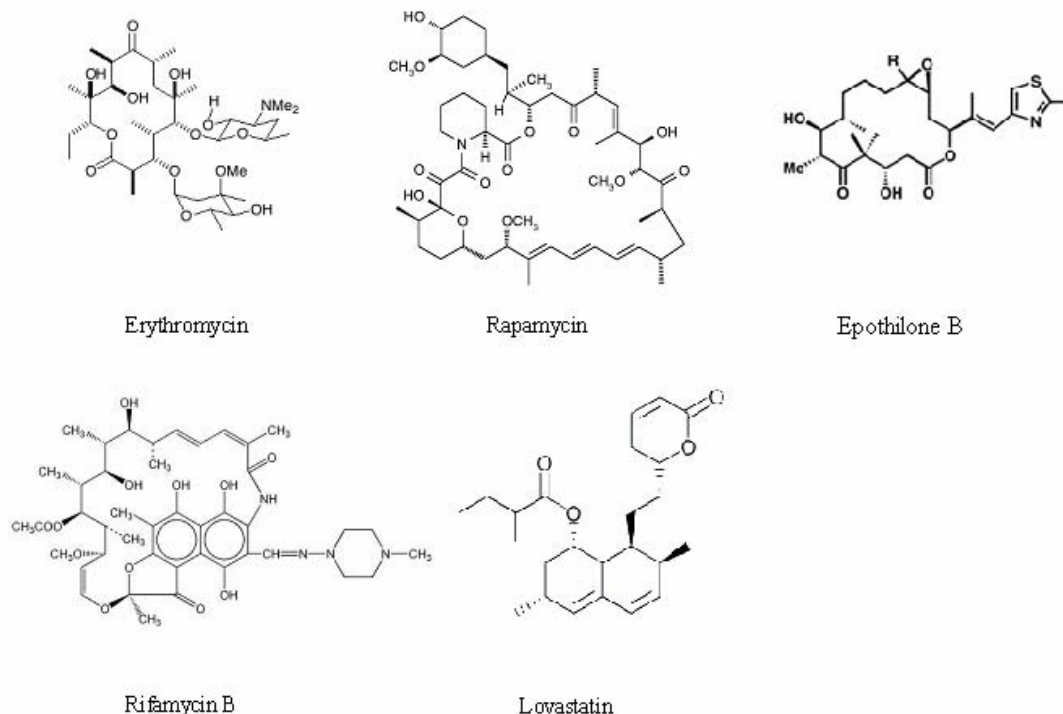
CHAPTER I

INTRODUCTION

I.A Polyketides

Polyketides are an important class of natural products that have complex forms and diverse biological functions. Possessing a wide range of biological activities, polyketides are often used for their antibiotic and pharmacological properties (Castoe 2007). In recent years, three major types of polyketide synthases have been discovered, iterative polyketide synthases, modular polyketide synthases, and mixed aromatic modular polyketide synthases. The iterative and aromatic polyketide synthases are found in fungal and bacterial strains (Bedford 1995; Tsoi 1995). Complex modular polyketide synthases are commonly studied with respect to engineering novel polyketides through modification of their modular architecture (Stein 2005). Several examples of natural polyketides are shown in the following figure.

FIGURE I.1 Examples of polyketide natural products (Floss 1999; Decker T. 2003; Altmann 2003 ; Ray 2004; Xie 2006)



The first polyketide shown, erythromycin, is an antibiotic isolated from *Saccharopolyspora erythraea*. This macrolide has also been used as a model polyketide for the generation of new antibiotics (Ray 2004). Rapamycin, often administered to patients receiving organ transplants, is another important polyketide. This polyketide has the activity to inhibit cell cycle progression and the growth of tumor cells (Decker T. 2003). Another therapeutic polyketide, Epothilone B, is a 16-membered macrolide that has shown anticancer activity. This polyketide promotes tubulin polymerization *in vitro* and the stabilization of microtubules against Ca^{+2} -dependent depolymerization (Altmann 2003). In 1959, the discovery of rifamycin made a large impact on the pharmaceutical

industry for patients suffering from mycobacterial infections. Rifamycin B has been administered to patients suffering from tuberculosis, leprosy, and mycobacterial infections related to AIDS/HIV (Floss 1999). Lovastatin, shown in figure I.1, is another therapeutic agent that has been used to assist in irregular cholesterol levels. Lovastatin has been administered to patients to increase the levels of low density lipoprotein activity leading to the decrease in cholesterol synthesis (Xie 2006).

The structural diversity of polyketides can be explained with an understanding of the mechanism of polyketide synthases. The polyketide synthases have been classified as Type I, Type II, and Type III, depending on their architecture, and biosynthetic mechanisms (Castoe 2007).

The Type I polyketide synthases are a diverse group of multifunctional enzymes. They contain the activities required to complete a cycle of β -keto acyl chain elongations. Within the Type I classification, there are two subgroups dividing the synthases by enzymatic activity and the type of host organism (Castoe 2007). Modular polyketides are produced in bacteria in which the synthases contains one or more modules. Each module is used once during the biosynthesis and is responsible for the production of one β -keto acyl group through a decarboxylation Claisen condensation between a malonyl derivative and the nascent polyketide chain. In iterative synthases, found in fungi, the modules are used repeatedly and are responsible for chain elongation through the formation of β -keto acyl groups (Castoe 2007).

The most common types of enzyme domains used in the Type I synthases are ketoacyl synthase (KS), acyl transferase (AT), ketoreductase (KR), dehydratase (DH),

enoyl reductase (ER), and thioesterases (TE). Minimal modules contain the KS, AT, and the acyl carrier protein (ACP) (Castoe 2007). This group is responsible for lengthening of the polyketide through the addition of acyl groups. The chains are then reduced using KR, DH, and ER. The polyketide is released from the PKS by a thioesterase, by hydrolysis or macrolactonization (Castoe 2007).

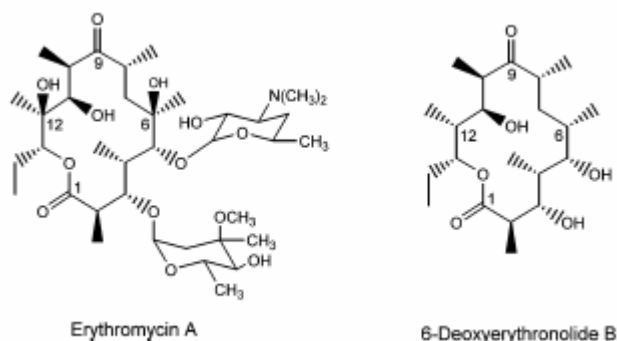
Type II and Type III synthases differ from Type I synthases in the position of the enzymatic activities. Type II synthases have arranged their synthases in separate polypeptides and use them repeatedly for chain elongation. In contrast, Type III synthases are arranged as single multifunctional modules and lack the phosphopantetheine (Castoe 2007). The phosphopantetheine is responsible for the transfer of the thiol from CoA from one active site to another.

I.B. Polyketide Biosynthesis

I.B.1. Erythromycin

The widespread use and studies of erythromycin (Figure I.2) have made it a model polyketide, for an understanding of polyketide biosynthesis. The polyketide has been used in therapeutic practices to inhibit the growth of Gram-positive bacteria (Lee 2004). Since the discovery of erythromycin in 1952, it has been used as a model compound in developing new antibacterial polyketides (Lee 2004). The structure of erythromycin is a polyketide core with two sugars, L-mycarose and D-desoamine, attached at the C-3 and C-5, respectively, shown in figure I.2 (Pereda 1998). The hydroxyl groups at C-6 and C-12 are a result of hydroxylation by cytochrome P450 enzymes.

FIGURE I.2 Erythromycin and 6-Deoxyerythronolide B (Lee 2004)

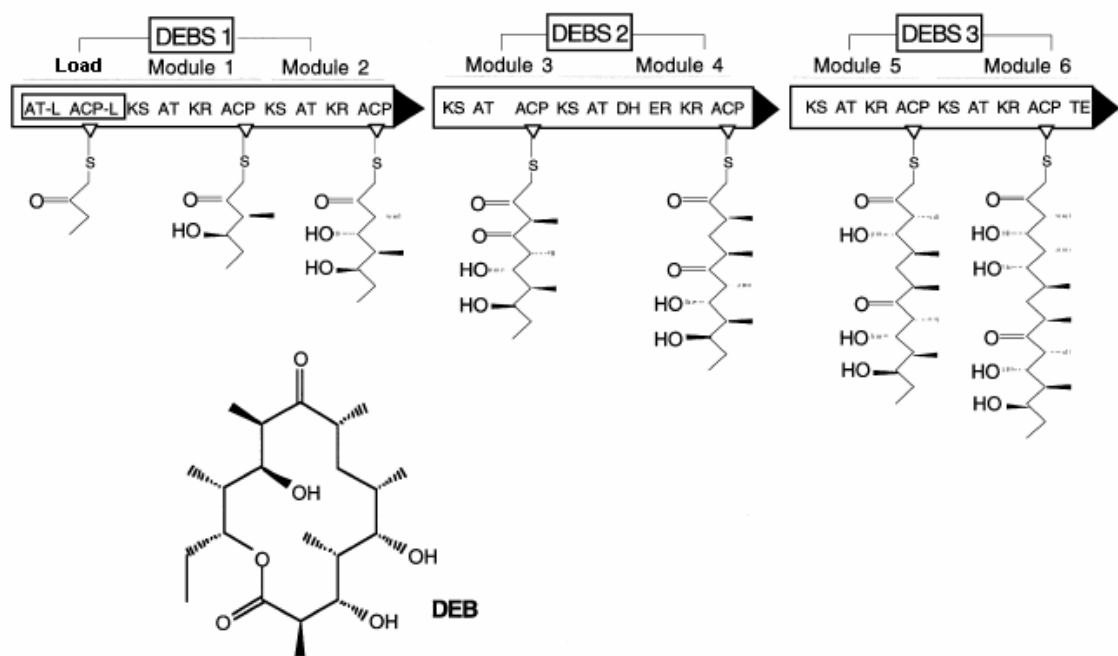


The precursor to erythromycin, 6- Deoxyerythronolide B, DEB, is synthesized from the 6- Deoxyerythronolide synthases, DEBS, which is a Type I modular polyketide synthase which contains 6 DEBS loading modules. Each module is responsible for the elongation of the chain, leading to the production of DEB (Pereda 1998).

The synthesis of DEB begins at the N-terminus in which the loading acyl transferase transfers a propionate from propionyl Co-A to the loading acyl carrier protein domain, shown in figure I.3 (Pereda 1998). From here, the propionate is transferred into module one in which a ketosynthase (KS1) extends the propionate by a Claisen condensation with (2S)-methylmalonyl-CoA. The β -ketone thus formed is reduced by a ketoreductase forming a β -alcohol (Pereda 1998). The following modules repeat the same Claisen reaction in which a total of six (2S)-methylmalonyl-CoA are added to the chain with some slight variation. Modules 1, 2, 5, and 6 leave alcohols on the final chain. However, module 3 does not contain the KR, while module 4 contains all three auxiliary domains, KR, DH, and ER. The lack of the KR in module 3 results in a ketone, and module 4 leaves a fully saturated group. After the chain is extended and processed

by module six, the polyketide is released by a macrolactonization catalyzed by the thioesterase (Pereda 1998). This produces the precursor to erythromycin, DEB.

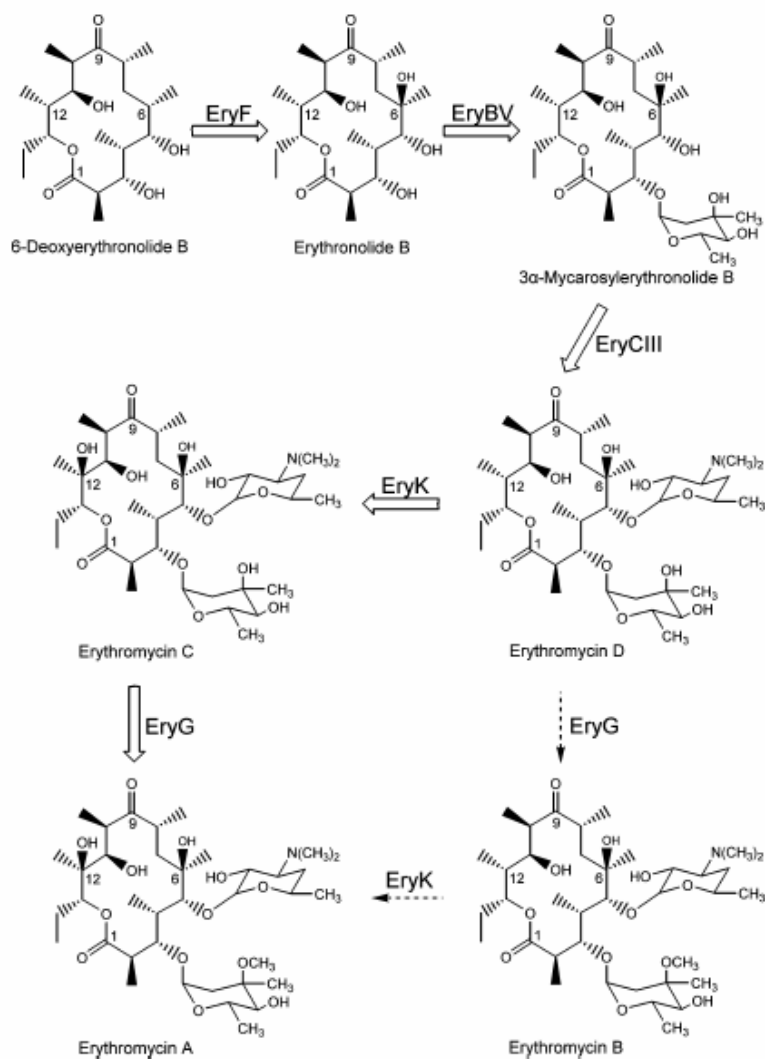
FIGURE I.3 Biosynthesis of 6- Deoxyerythronolide (Pereda 1998)



The precursor is hydroxylated by EryF at C6, followed by the addition of L-mycarose at C3 by EryBV, shown in figure I.4. The next step is the addition of D-desoamine to C5 by EryCIII (Lee 2004). At this point, erythromycin D can follow two different pathways. The preferred pathway in the synthesis of erythromycin A is the hydroxylation of C12 by EryK, a cytochrome P450, followed by the methylation of L-mycarose by EryG forming erythromycin A (Lee 2004). The second pathway involves the methylation of L-mycarose on erythromycin D and then the hydroxylation at C12 by

EryK. Although both pathways are possible, kinetic experiments show a 1000 fold preference for the first pathway (Lee 2004).

FIGURE I.4 Biosynthesis of Erythromycin from 6- Deoxyerythronolide (Lee 2004)



I.C. Bacillaene

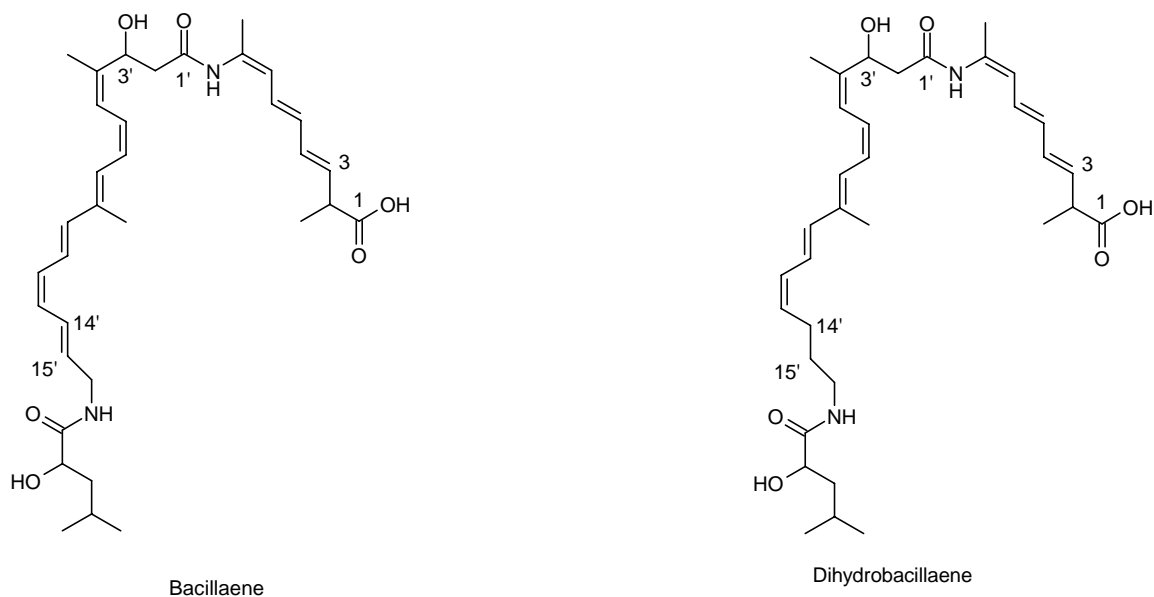
I.C.1 Bacillaene Biosynthesis

Bacillus subtilis has been responsible for the production of at least 3 polyketides. Furthermore, the completion of its genomic sequence was accomplished in 1997. The genome project resulted in the discovery of a 15-gene operon, *pksX*. The product of the *pksX* cluster was later identified as bacillaene (Butcher 2007).

Bacillaene was isolated in 1994 by Patel, et. al., from *Bacillus subtilis* and in 2006 by Chen et. al. from *Bacillus amyloliquefaciens* strain FZB 42 (Patel 1995; Chen 2006). The antibacterial activity of bacillaene has been studied because of its effectiveness in both Gram-positive and Gram-negative bacteria. Research has shown that bacillaene's activity when inhibiting protein synthesis for prokaryotic cells is high, however, it does not contain the ability to inhibit eukaryotic protein synthesis (Chen 2006).

Despite its first isolation in 1994, the structure of bacillaene was not solved until 2007. Any attempts to characterize bacillaene have been impeded by its instability in light and room temperature (Butcher 2007). Recently, the collaboration of two different techniques has made the structure of bacillaene available. After making *pksX* mutations of *Bacillus subtilis*, COSY and ROSEY NMR were used to monitor any changes exhibited on the spectra of partially purified bacillaene. After overlapping several spectra, the structures of bacillaene and dihydrobacillaene was finally determined, shown in figure I.5 (Butcher 2007).

FIGURE I.5 Structure of bacillaene and dihydrobacillaene (Butcher 2007)

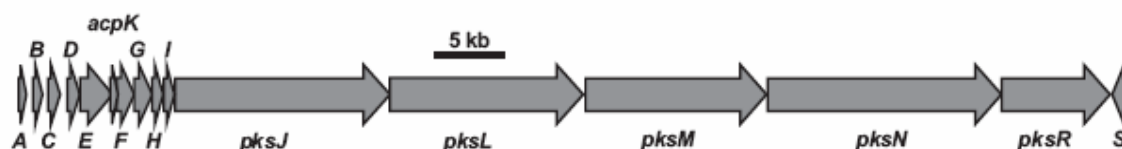


Gene knockout experiments have shown that the gene operon *pksX* is responsible for the production of bacillaene and dihydrobacillaene (Butcher 2007). Table I.1 and Figure I.6 detail the location of each gene within the operon and their proposed functions. The operon contains 5 polyketide synthases, two of which also contain NRPS domains. The acyltransferase activities needed by these synthases are believed to be supplied by the free-standing proteins, *pksCDE* (Chen 2006).

Table I.1 Function of Polyketide Synthases for Cluster *pksX* (Chen 2006)

Gene Name	Function based on Homology
<i>pksA</i>	Transcriptional Regulator
<i>pksB</i>	Zn-dependent hydrolase
<i>pksC-D</i>	Acyl Transferases (AT)
<i>pksE</i>	Acyl Transferase with flavin mononucleotide-dependent oxidase
<i>acpK</i>	Acyl Carrier Protein Transferase (ACP)
<i>pksF</i>	Ketosynthase
<i>pksG</i>	Hydroxymethylglutaryl (HMG)- CoA Synthase
<i>pksH-I</i>	Enol CoA Dehydrases
<i>pksJ-R</i>	Acyl Transferase-Less Type 1 Polyketide Synthases (PKSs)
<i>pksS</i>	Cytochrome P450 (hydroxylase)

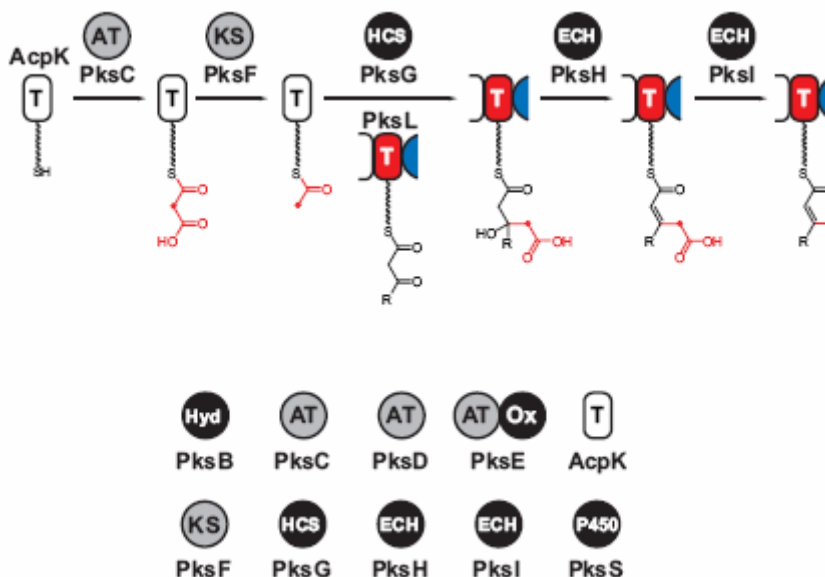
Figure I.6 Gene Cluster *pksX* (Chen 2006)



The biosynthesis of bacillaene, shown in figure I.7, begins with the gene *pksJ*. The first step is to load an α -hydroxyl-isocaproic acid onto the chain by *pksJ*. The next module provides the addition of a glycine by a NRPS condensation (c) domain . A malonyl group from malonyl CoA is then transferred onto the thiolation domain of the next module, and the KS domain adds it to the nascent chain, forming β -ketoacyl group. The ketone is reduced to an alcohol using ketoreductase. Using dehydratase, the alcohol is eliminated to an alkene. If the production of dihydrobacillaene is a result of the *pksX* gene cluster, it is believed that at this point, an unknown reductase is used to reduce the double bond (Butcher 2007). The most likely synthase responsible for the reduction that

branching carbon at C9'. The first step catalyzed by the subcluster is the addition of a malonyl CoA group to *acpK* by *pksC*. The malonyl group is decarboxylated by *pksF* to yield acetyl-*acpK* (Butcher 2007). The next gene, *pksG*, is expected to be the cause of the unusual subunit because of its homology with HMG-CoA synthase. An enoyl-CoA hydratase/isomerase is used to eliminate the hydroxyl group resulting in a double bond. The protein *pksI* decarboxylates the intermediate to form the final β -branch (Butcher 2007). After the formation of this unit, the chain is continuously elongated by remaining genes in the operon, *pksM*, *pksN*, and *pksR* complete the biosynthesis of bacillaene. The genes code for the addition of several malonyl units, followed by the reduction of β -keto acyl groups to alcohols. The chain is released from the PKS by the thioesterase (Butcher 2007).

FIGURE I.8 Subcluster within *pksL* (Butcher 2007)

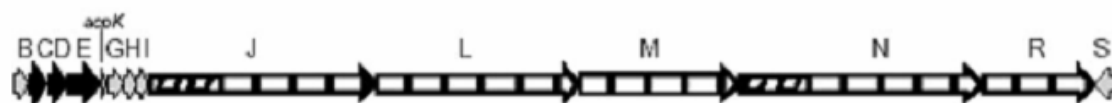


A second species that has been known to produce bacillaene is from *Bacillus amyloliquefaciens*, strain FZB42 (Chen 2006). This strain codes for three different gene clusters, in which one large cluster, *bae*, has been shown to be required for the biosynthesis of bacillaene in this species. The cluster is arranged nearly identically as the *pksX* from *B. subtilis*. The high degree of homology seen suggests that there is a common ancestor of the *pks/bae* cluster (Chen 2006). Figure I.9 and Table I.2 detail the function and position of each gene within the operon.

TABLE I.2 Function of Polyketide Synthases for Cluster *bae* (Chen 2006)

Gene Name	Function based on Homology
<i>baeB</i>	Zn-dependent hydrolase
<i>baeC-E</i>	Acyl Transferases (AT)
<i>baeF</i>	ACP
<i>baeG</i>	Hydroxymethylglutaryl (HMG)- CoA Synthase
<i>baeH-I</i>	Enol CoA Dehydrases
<i>baeJ-R</i>	Acyl Transferase-Less Type 1 Polyketide Synthases (PKSs)
<i>baeS</i>	Cytochrome P450 (hydrolase)

FIGURE I.9 *bae* gene cluster (Chen 2006)



A common gene found within both *pksX* and *bae*, is *pksS*, which is transcribed in *trans* from the other genes. Through sequence homology, *pksS* has been identified as a cytochrome P450. As a cytochrome P450, it is hypothesized that *pksS* acts as a hydroxylase in the polyketide biosynthesis by those two *Bacilli*.

By identifying *pksS* as a cytochrome P450, it will be possible to apply data to other novel polyketide systems. This can lead to determination and characterization of other polyketides with therapeutic capabilities. Also, information relating to the gene cluster that produces bacillaene could lead to more information concerning the potency and functionality of the polyketide. Data recorded on the functionality of *pksS* as a

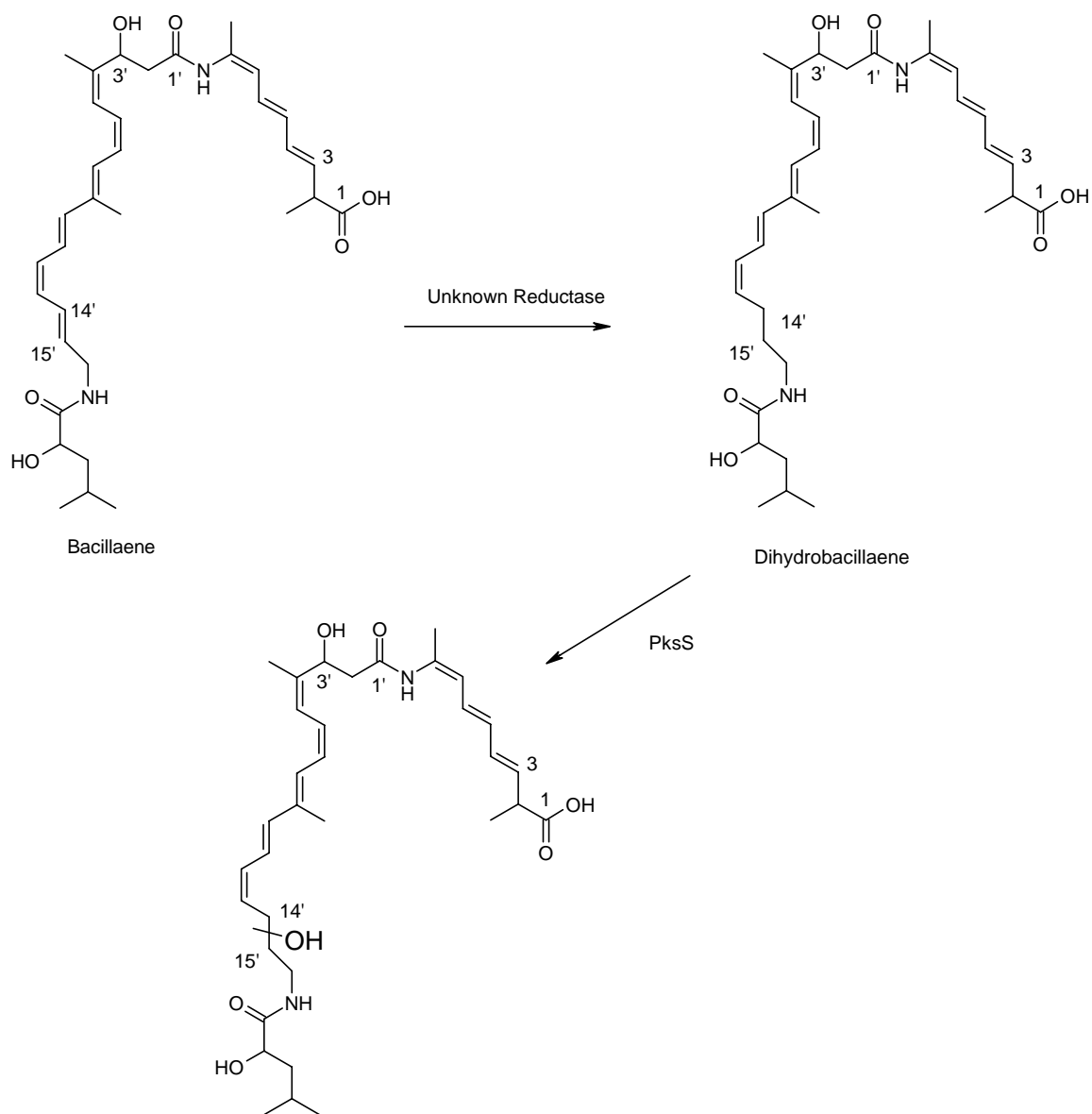
cytochrome P450 will be applied to developing a richer database of information for the field.

I.C.2. Role of PksS in the polyketide synthesis of bacillaene

Although the actual role of PksS in the polyketide biosynthesis of bacillaene has not yet been determined, sequence homology has allowed for the development of two hypothetical roles. Using sequence homology, PksS has been hypothesized to be a cytochrome P450, in which it acts as a hydroxylase.

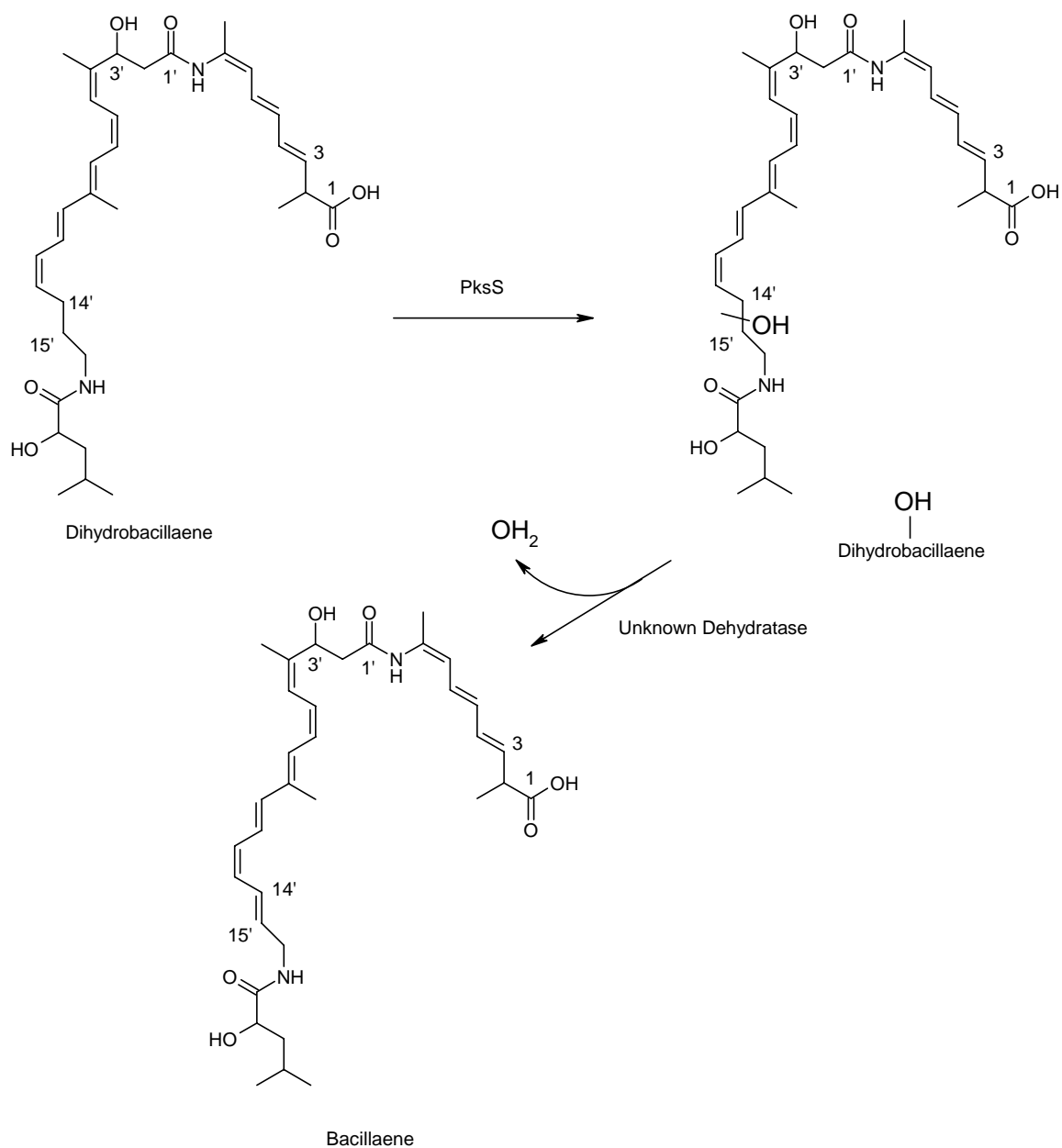
The first hypothesis assumes that an unknown reductase, possibly *pksE*, reduces bacillaene to dihydrobacillaene. Once dihydrobacillaene is produced, *pksS* hydroxylates C14 forming an alcohol group. The reaction is shown in figure I.10.

FIGURE I.10. Hypothesis 1 for the role of *pksS* in polyketide synthesis



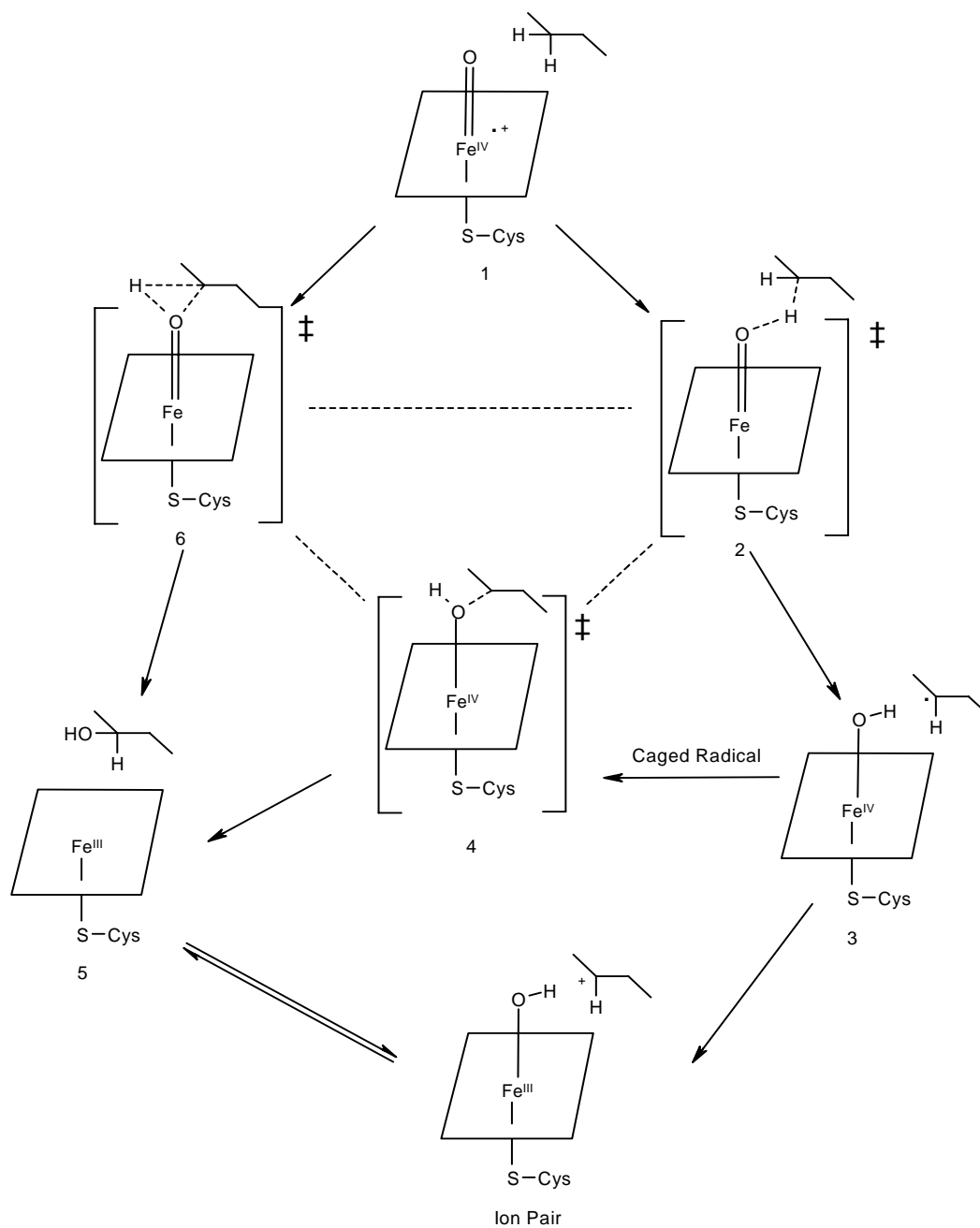
The second hypothesis assumes that dihydrobacillaene is produced first by the *pksX* polyketide synthases. After the production of dihydrobacillaene, it is possible that *pksS* hydroxylates the polyketide forming a hydroxylated version of dihydrobacillaene. The removal of water by some unknown dehydratase would form bacillaene, shown in figure I.11.

FIGURE I.11. Hypothesis 2 for the role of *pksS* in polyketide synthesis



Although the two hypotheses for the role of *pksS* occur at different locations of the polyketide biosynthesis, the pathways reactions expected for *pksS* are expected to be quite similar. We believe that PksS, a cytochrome P450, is brought to the Fe (V) state by a catalytic cycle involving a reductase that is well-known for cytochrome P450s (Ortiz de Montellano 2005). When the iron center comes into contact with the C14/C15, an alkane, the oxygen removes a hydrogen, producing a radical on C14'. This radical then forms a bond with the alcohol group, eventually removing the substituent from the heme. This generates a hydroxyl group on C14 and a ferric center of the cytochrome P450. The mechanism for this reaction is shown below in figure I.12 (Ortiz de Montellano 2005). Because the mechanism of hydroxylation has not yet been positively identified, several possible pathways to generate the hydroxyl group have been included in the figure.

FIGURE I.12. General reaction of PksS in polyketide synthesis (Ortiz de Montellano 2005)

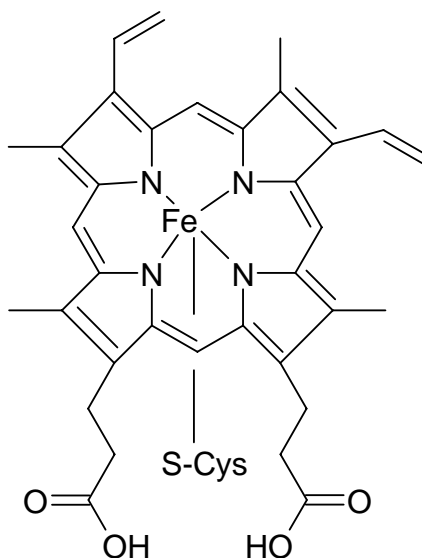


I.D. Cytochrome P450

Cytochrome P450 is a large class of enzymes that are responsible for the catalysis of many important oxidation reactions. Although the mechanisms of cytochrome P450s are well understood, several questions remain unanswered about the selectivity of the hydroxylation of the hydrocarbon. The main interest in cytochrome P450s is their ability to react with highly inert compounds, such as hydrocarbons (Carey 2003). Although these questions have begun to be answered, information determined here could lead to identifying the mechanism for the reactivity of cytochrome P450s.

Their structure is characterized by a signature iron center surrounded by a porphyrin ring, shown in figure I.13 (Berg 2002). This iron center is the reaction center in cytochrome P450 reactions. During a typical cytochrome P450 reaction, molecular oxygen is used to activate the iron. At a resting state, the iron center has an oxidation state of III (Berg 2002). Through action of a reductase, the iron center is reduced to II at which point molecular oxygen bonds as well as several other small ligands including carbon monoxide. The binding of carbon monoxide leads to a shift in its Soret Peak from 420 nm to 450 nm. This shift is the trademark reaction in identifying cytochrome P450s in biological systems (Ortiz de Montellano 2005).

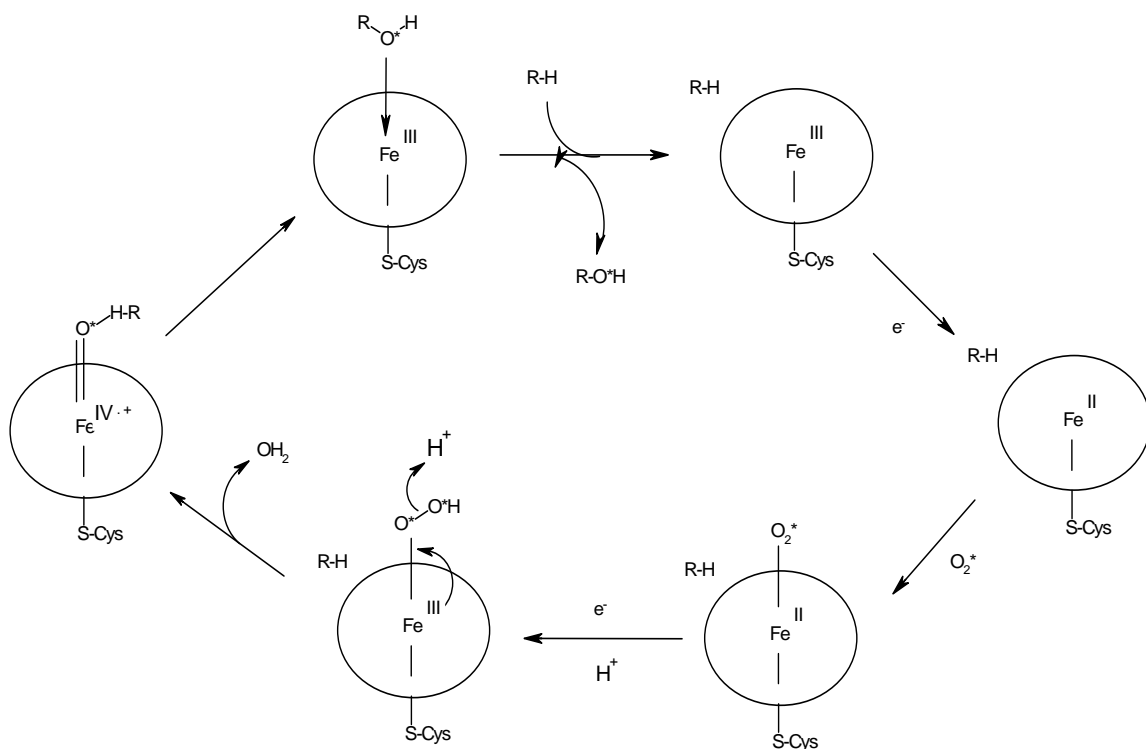
FIGURE I.13. Iron (III) protoporphyrin IX with a cysteine ligand



The general activation of molecular oxygen and transfer by a cytochrome P450 is shown in figure I.14. The mechanism for the activation has been determined through the monitoring of intermediate states by spectroscopic techniques. The activation of oxygen is begun through the binding of a substrate, identified as R-H, to the cytochrome P450 enzyme (Ortiz de Montellano 2005). The enzyme then accepts an electron from a reductase, resulting in the reduction of a ferric center to a ferrous center, at which point molecular oxygen binds. The ferrous-oxy complex is further reduced through the addition of another electron forming a Fe (III)-hydroperoxy complex (Ortiz de Montellano 2005). The peroxy-bond is cleaved by protonation of the terminal oxygen, releasing water and forming the iron IV-oxo porphyrin π -cation radical intermediate. The

hydroxylated product, once formed, is then released and the cytochrome P450 is regenerated to its Fe (III) state (Ortiz de Montellano 2005).

FIGURE I.14. Catalytic cycle for oxygen activation and transfer by cytochrome P450, molecular oxygen is represented by and asterisks (Ortiz de Montellano 2005)



The highly active centers are also known to catalyze several reactions used in polyketide synthesis and other metabolic pathways. A list of common reactions catalyzed by cytochrome P450s is listed in Table I.3. Two of the most common types of reactions in polyketide metabolism are the hydroxylation of hydrocarbons and the

epoxidation of alkenes (Bernhardt 2006). The hydroxylation of hydrocarbons are more commonly seen in polyketide synthesis and will be a major focus of this work.

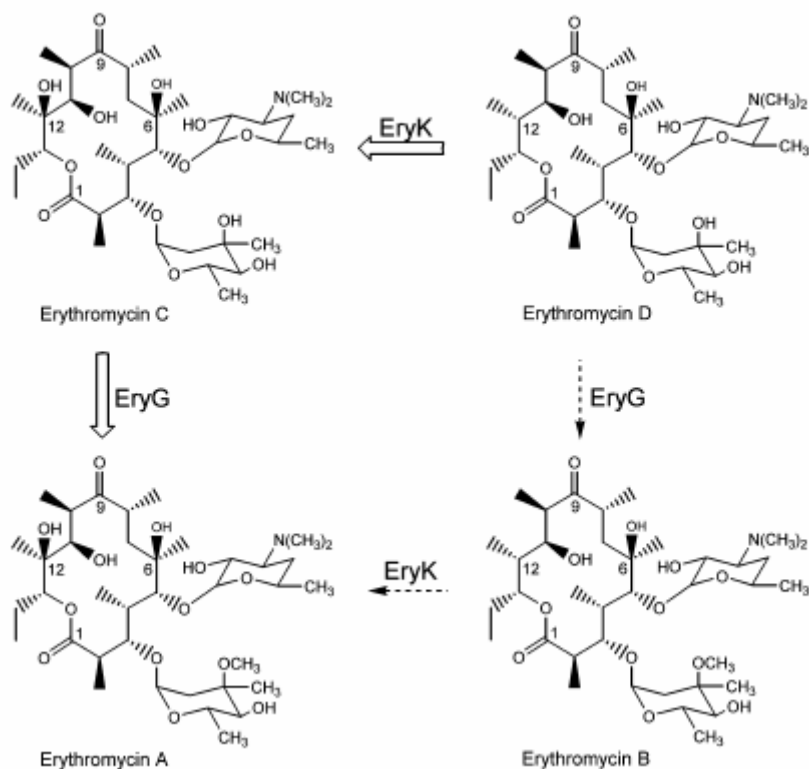
TABLE I.3. Possible cytochrome P450 reactions (Bernhardt 2006)

Hydrocarbon hydroxylation
S-Oxidation
Oxidative deamination
Oxidative dehalogenation
Aldehyde and Alcohol oxidations
Dehydrogenation
Dehydrations
Reductive dehalogenations
N-Oxide reduction
Epoxide reduction
Reductive β -scission of alkyl peroxides
Isomerizations
Alkene epoxidation
Alkyne oxygenation
Arene epoxidation
Aromatic hydroxylation
N-Dealkylation
S-Dealkylation
O-Dealkylation
N-Hydroxylation
N-Oxidation
NO reductions
Oxidative C-C bond cleavage

The diversity of polyketides is attributed to the diverse architectural variety of polyketide synthases (PKS). Following the biosynthesis of a polyketide core by the PKS, the products are modified by other enzymes, in which the compound undergoes various types of reactions, such as oxidation, methylation, and acylation (Lee 2004). The

oxidation of polyketides is often done through hydroxylations at aliphatic centers by cytochrome P450 monooxygenases. A good example of P450s involved in polyketide biosynthesis is the erythromycin C-12 hydroxylase, EryK, shown in figure I.15 (Lee 2004).

FIGURE I.15. The involvement of EryK in the biosynthesis of erythromycin A (Lee 2004)

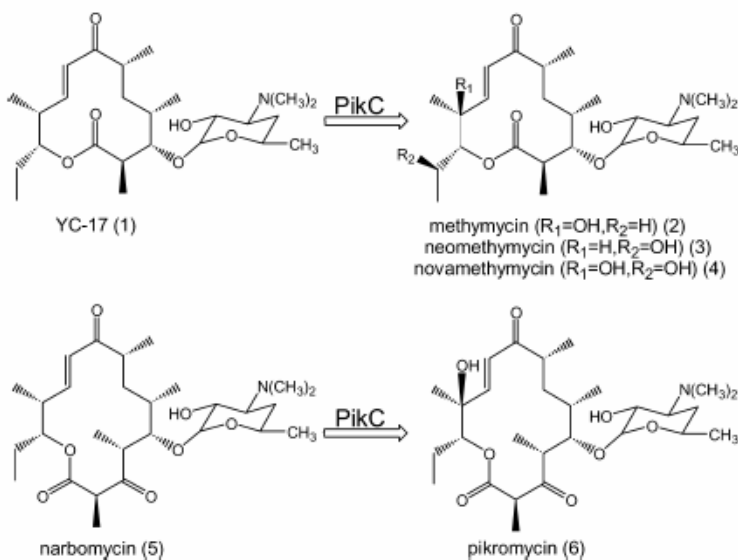


This pathway represents an important role that P450s play in the biosynthesis of polyketides. As many cytochrome P450s do, EryK is able to catalyze the hydroxylation of a specific aliphatic site on the natural product. As seen in figure I.15, EryK is able to

catalyze the hydroxylation of Erythromycin B, but the preferred pathway is the hydroxylation of Erythromycin D to Erythromycin C (Lee 2004).

Another polyketide hydroxylase, PikC, is also a well known cytochrome P450. This hydroxylase has the ability to catalyze the hydroxylation of two structurally different substrates, YC-17 and narbomycin. The flexibility that PikC shows during the hydroxylation of the substrates has been useful for the construction of novel macrolides and ketolides (Lee 2004). Figure I.16 shows that PikC can hydroxylate narbomycin through C-12 hydroxylation to form pikromycin. PikC is also able to catalyze the hydroxylation of either C-10 or C-12 YC-17 to form methymycin and neomethymycin, respectively (Lee 2004).

FIGURE I.16. Hydroxylation of YC-17 and Narbomycin by PikC (Lee 2004)



The diversity of polyketides is attributed to the structural organization of polyketide synthases, and tailoring reactions, which occur after the synthases complete the polyketide. The polyketide synthases assemble polyketides by a similar mechanism as fatty acid synthesis. This homology is important when trying to determine the pathway each synthase undergoes. Following polyketide synthesis, the polyketides are often further modified by other tailoring enzymes. Cytochrome P450s are a common type of tailoring enzyme that are responsible for the hydroxylation of many polyketides. We believe that PksS is a cytochrome P450 and will hydroxylate dihydrobacillaene, bacillaene, or both. The following two chapters outline our work towards the purification and characterization of this protein.

CHAPTER II

CLONING, OVEREXPRESSION, AND PURIFICATION OF PKSS

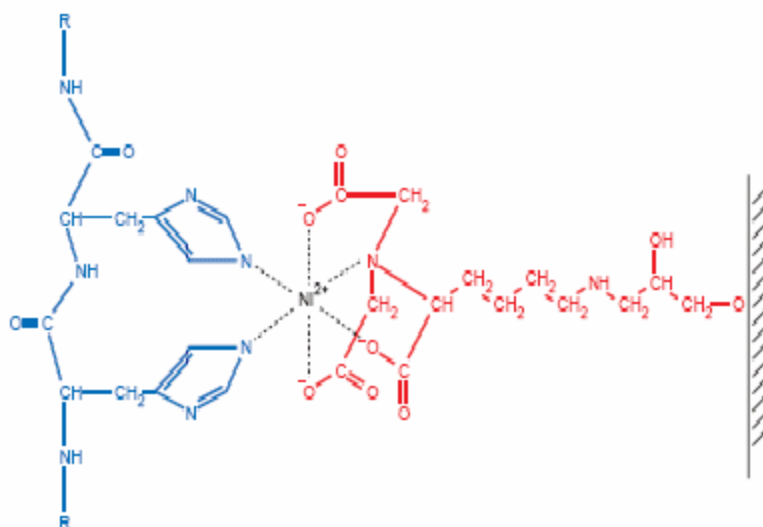
II. A. Production of pQE60/*pksS*

II.A.1. Goals

In order to determine the function of PksS in bacillaene biosynthesis, *pksS* must first be cloned into a vector suitable for the efficient overexpression of PksS. The vector pQE60 was chosen for its known effectiveness in directing protein synthesis and for incorporating His-Tag sequences into recombinant proteins. This vector controls protein overexpression by using an optimized T5 promoter (Gu 1994; Flachmann 1996; Noji 1997; Greiner 1998). The T5 promoter is also controlled by the LacI repressor, which is supplied in *trans* by another plasmid called pREP4. The LacI repressor is a well-documented means to rationally control the transcription of the gene through the addition of isopropyl- β -D-thiogalactoside, an artificial inducer (Gu 1994; Flachmann 1996; Noji 1997; Greiner 1998). The cloning of *pksS* into the pQE60 vector is accomplished by utilizing the restriction sites, *NcoI* and *BglIII*, located in the vector and incorporated into the insert through PCR primers. Following the digests, the target gene and vector are connected by T4 ligase. The T4 ligase catalyses the formation of a phosphodiester bond between a 5' phosphate group and a 3' hydroxyl group (Engler 1982 ; Remaut 1983; Sambrook 1989).

The ligation products are obtained by transforming the crude reaction into competent *E. coli* and grown under antibiotic selection. The gene of interest can then be overexpressed by induction with IPTG to produce the target protein, PksS. After the successful production of PksS, a C-terminal His₆ tag, which is incorporated into the protein by the cloning vector, will be used for affinity purification. When combined with a nickel-nitrilotriacetic acid (Ni-NTA) column, the imidazole side chains in the protein's His-tag is expected to form a complex with the Ni⁺², (Figure II.1) ultimately allowing for the removal of other proteins and impurities. The Ni-NTA column occupies four of the six binding sites of the nickel, leaving two that can be occupied by adjacent imidazoles on a protein (Anderson 1997; Lebrón 1998 ; West 2000). After several washes of increasingly higher concentrations of imidazole, the protein is eventually eluted from the column.

FIGURE II.1. Ni-NTA column purification of His-Tagged proteins (Anderson 1997; Lebrón 1998 ; West 2000)



II.B. Results

II.B. 1. Preliminary data of cloning *pksS*

In prior work, Dr. Jason Reddick and Dr. Gregory Raner attempted to clone *pksS* using two different sets of primers, Set 1 and Set 2. The primers used during each polymerase chain reaction throughout this research are reported in Table II.1. Upon completion of PCR, electrophoresis showed that the primers were designed correctly so they continued to clone *pksS* with this PCR product. After purification of the PCR product, they proceeded to ligate the insert into the plasmid, pET-28a. After several unsuccessful attempts to express the protein they decided to try new primers.

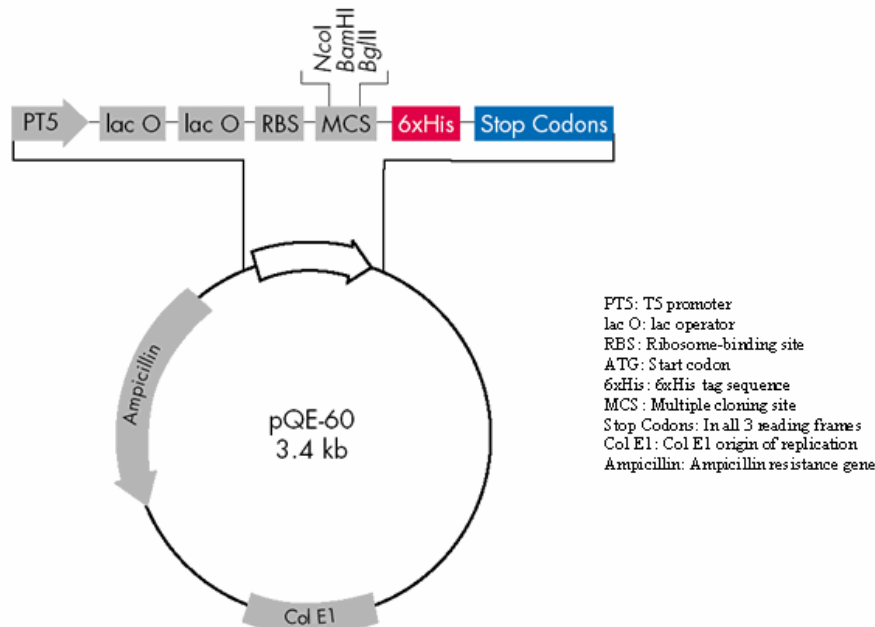
II.B.2. Cloning *pksS* with vent polymerase

In an attempt to clone *pksS*, several primers were designed incorporating *NcoI* and *BglII* restriction sites at the ends. These primers were combined with Vent polymerase, a polymerase with 3'—5' proofreading exonuclease activity, and the genomic DNA from *Bacillus subtilis* 168, as a template (de Noronha 1992).

Through PCR, *pksS* was amplified from *Bacillus subtilis* strain 168 genomic DNA. The gel electrophoresis showed an intense band between 1100-1200 bp, demonstrating a successful PCR. Upon completion, *pksS* was purified and prepared for the restriction digest/ligation step utilizing the *NcoI* and *BglII* restriction sites.

An agarose gel showed that the restriction digest was successful, therefore, the *pksS* insert and pQE60 plasmid, shown in figure II.2, were ligated using T4 ligase. In three separate reactions, different concentrations of insert were used to ensure that optimal ligation conditions were approached.

FIGURE II.2. pQE60 vector map showing restriction site locations (Anderson 1997; Lebrón 1998 ; West 2000)



Following the ligation, the mixture was transformed into competent DH5 α (*E. coli*) cells. Once the plasmids were transformed into the host cell, the cells were plated on agar plates containing ampicillin. Cells containing 2 μ L and 5 μ L volumes grew after a 24 hour incubation period. Plasmids from the four starter cultures were inoculated with cells and were grown overnight at 37 °C with kanamycin and ampicillin. The four cultures were then purified the following day.

Following the purification, the plasmids were screened using *Taq* (HotMaster) polymerase because sequence fidelity was not required. Following the PCR, a gel was run to determine if *pksS* had been successfully cloned into the pQE60 plasmid. The agarose gel showed a band of approximately 4 kb, which is 3 kb longer than the desired

gene. Unsure of the cause of this increase, a restriction digest was run on the purified plasmid DNA to determine the sizes of the gene. The digest showed several bands of incorrect sizes. *Therefore, the ligation was deemed unsuccessful.*

After the failure of the previous experiments, the cloning process was restarted from PCR using the upstream primers pksS pQEUP3 and pksS pQEUP4, shown in Table II.1, and the downstream primer pksS. The agarose gel used to determine if the gene was amplified showed one band for pksS pQEUP3 at the correct size, 1.12 kb, and three bands for pksS pQEUP4. PCR was reattempted several times for pQEUP4 to the same effect. *At this point, it was determined that the upstream primer pksS pQEUP3, was effective and pksS pQEUP4 was not effective for PCR.*

TABLE II.1. List of primers used in PCR

Designated Name	Sequence 5'—3'
Set 1 UP	5'- TACCCTATTAGGCGGCTAGCATGCAAATGGA -3'
Set 1 DN	5'- TCATCAGGTGCAACCTCGAGTTCAATATCAGGAAAT - 3'
Set 2 UP	5'- GCAATTTGATTCATCTATTTACCCTATTAGGCGGCTAGC -3'
Set 2 DN	5'- CTCCATTGAATGTCATCAGGTGCAACCTCGAG -3'
pksS pQE-UP1	5' - TATCCATGGAAATGGAAAAATTG - 3'
pksS pQE-UP2	5' - ATGCCCATGGAAATGATG - 3'
pksS pQE-DN	5' - TATAGATCTGGAAATCTCTTCAAAACG - 3'
pksS pQEUP3	5' - TATCCATGGAAATGGAAAAATTGATGTTTCATCCG - 3'
pksS pQEUP4	5' - ATGCCCATGGAAATGATGTTTCATCCGCATGG - 3'
pksS pQEDN2	5' - TATAGATCTGGAAATCTCTTCAAAAGCTGC - 3'
pksS UP SAA	5' - CGAGGACCATGGAAATGGAAAAATTG - 3'
pksS DN2 SAA	5' - CAAGTTCTAGATCTGGAAATCTCTTCAAAAGC - 3'
pksS long DN SAA	5' - GGGGCGTTTTGAAGATCTTTTGAAAGTGAAACAGG - 3'

II.B.3. Topo cloning

In another attempt to clone *pksS*, a PCR was run using the upstream primer pksS pQEUP3 and the downstream primer pksS pQEDN2. The product was purified and then cloned into pBAD using topoisomerase cloning technology. After ligating the *pksS* into the pBAD vector, the plasmid was transformed into “One Shot Top10 Competent Cells,” plated onto an ampicillin/LB plate, and grown overnight at 37 °C. Three colonies from two plates were used to inoculate starter cultures containing ampicillin. Following the incubation, the colonies were purified and were digested using the restriction sites *NcoI*

and *Bgl*III. *Following the restriction digest, gel electrophoresis revealed that the cloning of pksS was unsuccessful by the lack of bands.*

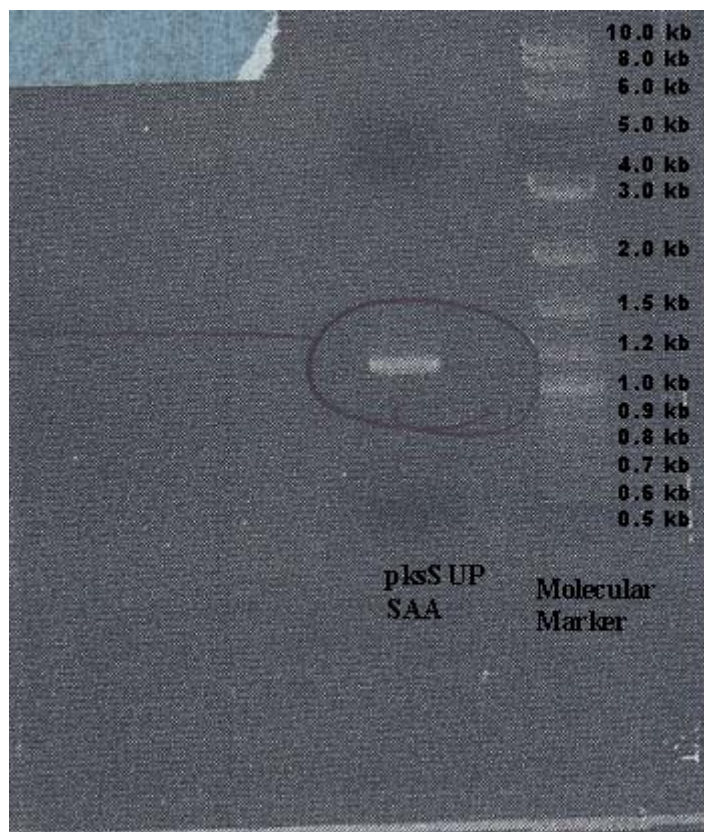
II.B.4. Cloning *pksS* using Phusion polymerase

In another attempt to successfully clone the *pksS* gene, a new polymerase, Phusion High-Fidelity DNA Polymerase, was used. The PCR was run using the upstream primer pksS pQEUP3 and the downstream primer pksS pQEDN2. After completion of the PCR, gel electrophoresis was used to determine if *pksS* was amplified.

The gel revealed that the amplification of the gene was unsuccessful.

After the previous failure to clone *pksS*, a new upstream primer, pksS UP SAA and downstream primer, pksS DN SAA were designed. Following gel electrophoresis, shown in figure II.3, the product was purified. This PCR product and cloning vector, pQE60, were then digested with *Nco*I and *Bgl*III. The digested material was ligated using three different concentration of insert as before. After incubation, the ligation mixtures were transformed into a new host cell, M15[pREP4] and then plated onto agar plates containing ampicillin and kanamycin. The following day, transformants were used to inoculate starter cultures, which were grown overnight. The plasmids were purified from the starter cultures using the “QIAprep Spin Miniprep Kit.” After purification, the plasmids were digested by *Nco*I/*Bgl*III and analyzed by agarose gel electrophoresis. *The gel revealed that the pksS gene was successfully cloned into pQE60.*

FIGURE II.3. Agarose gel of *pksS* pQE UPSAA and *pksS* pQE DN SAA



After the successful cloning of *pksS* into pQE60, the plasmids were retransformed into M15[pREP4] and plated. The next step was to overexpress *pksS* using the plasmid. The cells were grown in terrific broth (TB) media containing 30 µg/ml kanamycin and 25 µg/ml ampicillin. After a two day incubation period at room temperature, the cells were induced with 0.63 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) (Phillips 1998). After induction, the culture continued to grow at room temperature with shaking at a speed of 70 rpm for 24 hours (Phillips 1998). The cells were harvested by centrifuging the cultures and then stored at -80.0°C.

Following the overexpression procedure, a Ni-NTA column was used to purify PksS. During the chromatography, aliquots of the protein were collected from each step in the imidazole gradients. The samples were analyzed by SDS-PAGE (Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis) to check if the protein had been extracted. At this point, it was determined the pksS had not been expressed, due to the absence of a band with a molecular weight of 46 kDa in any gel sample.

II.B.5. Cloning, overexpression, purification with pLys RARE plasmid

It is quite possible that the protein is not being produced due to a presence in the *pksS* gene of codons that *E. coli* rarely uses. In fact, there are 3 consecutive codons in the middle of the gene which are rarely used in *E. coli*. By coexpressing pQE60 and *pksS* with another plasmid, pLys RARE, it would be possible to express codons not normally used during overexpression. The pLys RARE plasmid was transformed into M15[pREP4] already replicating pQE60/*pksS*, and then the cells were plated. Following incubation, colonies were used to inoculate starter cultures. Upon reaching optical density, $OD_{595}=0.50-0.60$, the cultures were used to inoculate TB media. After growing for two days, the cultures were induced with IPTG. Samples were then removed every hour for five hours to check for cytochrome P450 expression using the carbon monoxide complex spectroscopy method (Phillips 1998). *The assays did not display peaks corresponding to a cytochrome P450, therefore, it was determined that there was no P450 present in the culture.*

Following purification, SDS-PAGE was used to determine if any protein of ~46 kDa was expressed during the growth. Protein collected during purification revealed that PksS had not been produced.

In another attempt to overexpress *pksS*, other transformants that tested positive for *pksS*, were used to inoculate several starter cultures. Following incubation, the starter cultures were used to inoculate Luria-Bertani (LB) cultures. After reaching optical density of 0.5-0.6 at 595 nm, the cultures were induced with IPTG and grown for three hours.

Throughout the overexpression, samples were taken for SDS-PAGE analysis. A faint band around the correct size was seen in some of the samples. To increase the production of PksS, the media was prepared with 2% glycerol to enrich the culture. The LB was inoculated using starter cultures containing colonies from a previous transformation and grown according to standard protocol. Following incubation, samples were analyzed by SDS-PAGE. ***No bands at 46 kDa were detected and a new method was chosen.***

The next attempt to overexpress *pksS* utilized the introduction of 1% dextrose to the LB media. Starter cultures were grown using colonies from the previous transformation and then used to inoculate the media. Following induction of the media, samples were removed from the culture and analyzed by SDS-PAGE. Also, Ni-NTA purification fractions obtained were analyzed by SDS-PAGE. ***The gels resulted in inconclusive information and the procedure was repeated again.***

After several unsuccessful attempts to overexpress PksS, M15[pREP4] transformants containing pksS/pQE60 were plated and made competent using 50 mM CaCl₂. The pLys RARE plasmid was transformed into the cells and grown on an agar plate. This transformation resulted in two different sizes of colonies, large and small. Both sizes of the colonies were used to inoculate starter cultures. Following their incubation, the starter cultures were used to inoculate LB containing dextrose. After inducing the media, samples were removed to determine by SDS-PAGE if PksS was being produced. Although the results from the culture were inconclusive, crude extracts were subjected to Ni-NTA chromatography. During the purification, samples were removed from the washes and then analyzed by SDS-PAGE. ***The gel showed no conclusive evidence of His-Tagged pksS being expressed.***

II.B.6. Sequencing pksS

Due to several unsuccessful attempts to clone and purify PksS, pQE60/pksS plasmid was sequenced by standard dye-termination DNA sequencing (SeqWright, Inc.). ***The sequence data indicated that our plasmid lacked Guanosine 1111 reported for pksS from the genomic sequence for Bacillus subtilis.*** The published sequence and our experimental sequence of pksS is shown in figure II.4. The location of the missing nucleotide is highlighted.

FIGURE II.4. Alignment of published sequence of *pksS* to data reported from sequencing

Published <i>pksS</i>	1	atgcaaatggaaaaattgatgtttcatccgcatggtaaagagtttcatcacaaatcctttt	60
Seq <i>pksS</i> insert	1	atgcaaatggaaaaattgatgtttcatccgcatggtaaagagtttcatcacaaatcctttt	60
Published <i>pksS</i>	61	tcagtttttaggacgatttagagaggaagagcccattcaccgatttgaattaaaacggttc	120
Seq <i>pksS</i> insert	61	tcagtttttaggacgatttagagaggaagagcccattcaccgatttgaattaaaacggttc	120
Published <i>pksS</i>	121	ggagccacatatccggcctggtaattacccgatac gatgattgtatggcctttttaaaa	180
Seq <i>pksS</i> insert	121	ggagccacatatccggcctggtaattacccgatac gatgattgtatggcctttttaaaa	180
Published <i>pksS</i>	181	gacaatcgaattacaagagacgtaaaaaatgtgatgaaccaagaacaaatcaaaatgctc	240
Seq <i>pksS</i> insert	181	gacaatcgaattacaagagacgtaaaaaatgtgatgaaccaagaacaaatcaaaatgctc	240
Published <i>pksS</i>	241	aacgttagtgaagatatcgattttgtatccgatcatatgctggcaaaagacacacctgac	300
Seq <i>pksS</i> insert	241	aacgttagtgaagatatcgattttgtatccgatcatatgctggcaaaagacacacctgac	300
Published <i>pksS</i>	301	cataccgcctgagatcacttggtcatcaagcatttactccccgaaccattgaaaatctg	360
Seq <i>pksS</i> insert	301	cataccgcctgagatcacttggtcatcaagcatttactccccgaaccattgaaaatctg	360
Published <i>pksS</i>	361	cgcggcagcattgaacaaattgctgaacagcttttagatgaaatggaaaaagaaaaataa	420
Seq <i>pksS</i> insert	361	cgcggcagcattgaacaaattgctgaacagcttttagatgaaatggaaaaagaaaaataa	420
Published <i>pksS</i>	421	gcggatatcatgaaatccttcgcttccccctttgccttttattgttatatctgaattgatg	480
Seq <i>pksS</i> insert	421	gcggatatcatgaaatccttcgcttccccctttgccttttattgttatatctgaattgatg	480
Published <i>pksS</i>	481	ggaatcccaaaagaagatcggtcacagtttcaaactctggaccaatgcgatgggtgatacc	540
Seq <i>pksS</i> insert	481	ggaatcccaaaagaagatcggtcacagtttcaaactctggaccaatgcgatgggtgatacc	540
Published <i>pksS</i>	541	tctgaaggtaatagagagctgacaaatcaggcccttcgtgaatttaaagattatatcgct	600
Seq <i>pksS</i> insert	541	tctgaaggtaatagagagctgacaaatcaggcccttcgtgaatttaaagattatatcgct	600
Published <i>pksS</i>	601	aagctgatccatgacagaagaataaagccaaaagacgatttaacagcaaaacttgtgcat	660
Seq <i>pksS</i> insert	601	aagctgatccatgacagaagaataaagccaaaagacgatttaacagcaaaacttgtgcat	660
Published <i>pksS</i>	661	gctgaggaaaaacggcagcaagttaagcgaaaaagagctctattcgatgctgttcttgctc	720
Seq <i>pksS</i> insert	661	gctgaggaaaaacggcagcaagttaagcgaaaaagagctctattcgatgctgttcttgctc	720
Published <i>pksS</i>	721	gttgtagccggccttgaaacaactgttaacttactcggctcaggcaccctcgattgctg	780
Seq <i>pksS</i> insert	721	gttgtagccggccttgaaacaactgttaacttactcggctcaggcaccctcgattgctg	780
Published <i>pksS</i>	781	cagcacaagaaggaatgtgagaagctcaagcagcagcctgaaatgatcgctacagcggtt	840
Seq <i>pksS</i> insert	781	cagcacaagaaggaatgtgagaagctcaagcagcagcctgaaatgatcgctacagcggtt	840
Published <i>pksS</i>	841	gaagaattgctgcgatacacctcacctgtcggttatgatggcaaatcggtgggccatcgaa	900
Seq <i>pksS</i> insert	841	gaagaattgctgcgatacacctcacctgtcggttatgatggcaaatcggtgggccatcgaa	900
Published <i>pksS</i>	901	gactttacatataaggggcattcgatcaaaagaggagacatgatttttataggcatcgga	960
Seq <i>pksS</i> insert	901	gactttacatataaggggcattcgatcaaaagaggagacatgatttttataggcatcgga	960
Published <i>pksS</i>	961	tctgccaatcgcgaccgcaatttttttgagaaccccgaaatattaataataaatcggtcg	1020
Seq <i>pksS</i> insert	961	tctgccaatcgcgaccgcaatttttttgagaaccccgaaatattaataataaatcggtcg	1020
Published <i>pksS</i>	1021	cctaatagacatattttcttttggttttggtcatttctgcttaggagcgccctcttgcc	1080
Seq <i>pksS</i> insert	1021	cctaatagacatattttcttttggttttggtcatttctgcttaggagcgccctcttgcc	1080
Published <i>pksS</i>	1081	aggctggaaggccacattgcatttaaagcagcttttgaagagatttcc-----	1128
Seq <i>pksS</i> insert	1081	aggctggaaggccacattgcatttaaagcagcttttgaagagatttcc-----	1128



II.B.7. Using new primers, PKSS UP SAA and PKSS DN2 SAA

Due to the high adenine/thiamine content in the our downstream primer, it is possible that the primers may have incorrectly annealed and the guanosine was skipped during PCR. After receiving the sequencing information, new primers, pksS UP SAA and pksS DN2 SAA, were designed that would force the PCR to include G1111, if it was present. Briefly, the downstream primer had the complementary cytosine at the 3' end, and the PCR should work only if G1111 was present. After determining that the PCR yielded the correct sized product by gel electrophoresis, also shown in figure II.5, the product was purified and digested for cloning into pQE60.

FIGURE II.5. Agarose gel of PCR product using pksS UP SAA and pksS DN2 SAA



The restriction digest products, shown in figure II.6, were then ligated together using three different concentrations of insert. The ligation mixtures were transformed into M15[pREP4] cells and plated on agar plates. The resultant colonies were used to inoculate several starter cultures. Each culture was purified and then digested to verify the addition of the insert into the plasmid, shown in figure II.6. After revealing successful ligation products, a successful plasmid was retransformed into M15[pREP4] . For the purpose of sequencing and overexpression, the same colony was used to inoculate two starter cultures comprised of LB and dextrose. Following the incubation, the plasmid from one starter culture was purified and sequenced. The second culture was used to inoculate an overexpression culture. The culture was grown to optimal density and induced with IPTG. Subsequently, samples to be analyzed by SDS-PAGE were removed during the overexpression period and purification process. *The gel revealed that no PksS protein was being produced at any point.*

FIGURE II.6. Restriction digest of *pksS* and pQE60, Lane 1: Molecular Weight, Lane 3: Uncut pQE60, Lane 5: Cut pQE60, Lane 7: Cut *pksS*

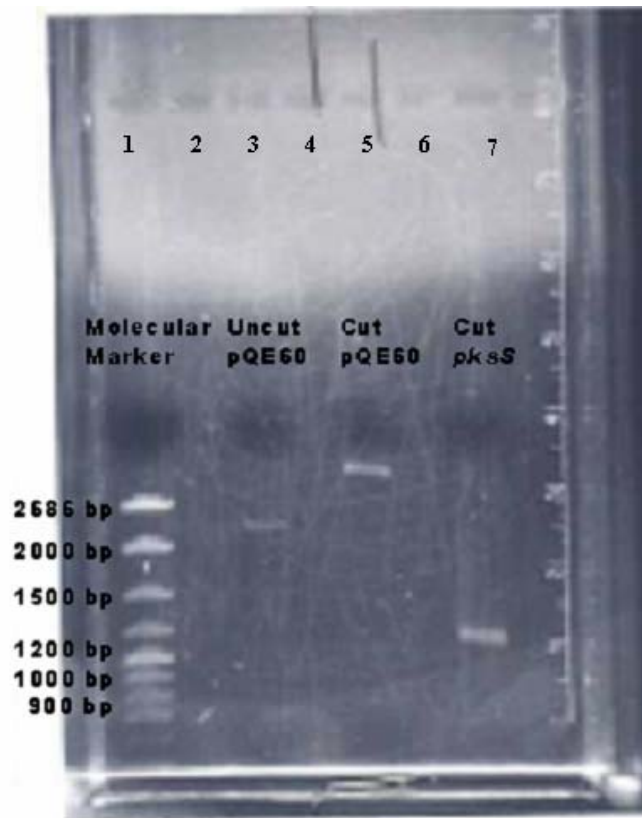
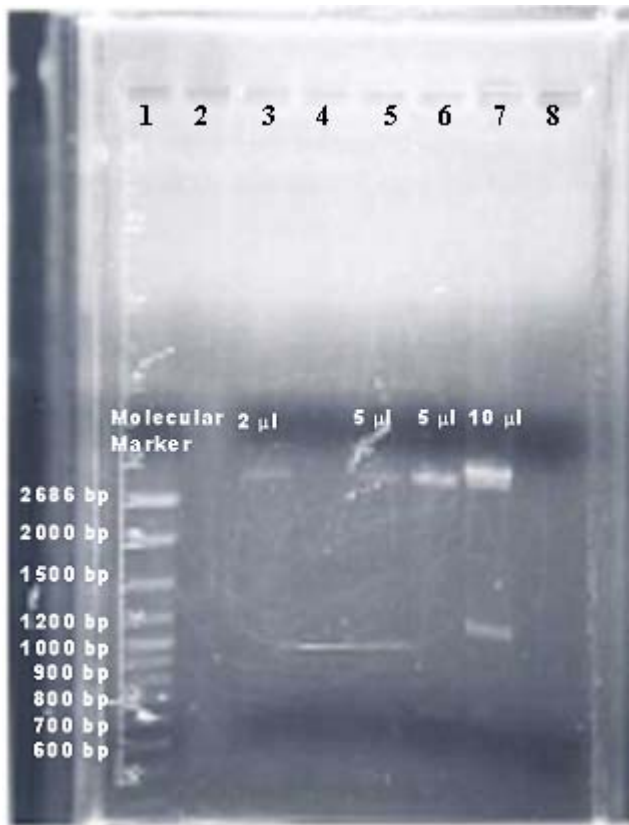


FIGURE II.7. Restriction digest of ligation reactions, Lane 1: Molecular Weight, Lane 3,4: 2 μ L insert: 1 μ L plasmid, Lane 5,6: 5 μ L insert: 1 μ L plasmid, Lane 7,8: 10 μ L insert: 1 μ L of plasmid



Despite using primers that would force the PCR reaction to include G1111, sequencing still revealed an absence of G1111. The best explanation for this is that the guanosine 1111 previously reported in the *Bacillus subtilis* sequence was a mistake. The polymerase used during our PCR, Phusion, is a proofreading polymerase, therefore it likely cut the theoretical G1111 complement cytosine, allowing the polymerase to continue DNA synthesis on the template. These results suggest that the published genome sequence for BS168 has an erroneous extra guanosine after A1110. Such an

error is a frame-shift error, therefore, the assigned stop codon is incorrect. *The genomic database was searched for the next in frame stop codon, and we determined that the true length of pksS was 1224 bp instead of 1128 bp, shown in figure II.8. The corrected amino acid sequence, which will be used for sequence homology is shown in figure II.9.*

FIGURE II.8. Corrected sequence of *pksS*

pksS corrected	1	atgcaaatggaaaaattgatgtttcatccgcatggtaaagagtttcatcacaaatcctttt	60
published pksS	1	atgcaaatggaaaaattgatgtttcatccgcatggtaaagagtttcatcacaaatcctttt	60
pksS corrected	61	tcagtttttaggacgatttagagaggaagagcccattcaccgatttgaattaaaacggttc	120
published pksS	61	tcagtttttaggacgatttagagaggaagagcccattcaccgatttgaattaaaacggttc	120
pksS corrected	121	ggagccacatatccggcctggtaattacccgatac gatgattgtatggcctttttaaaa	180
published pksS	121	ggagccacatatccggcctggtaattacccgatac gatgattgtatggcctttttaaaa	180
pksS corrected	181	gacaatcgaattacaagagacgtaaaaaatgtgatgaaccaagaacaaatcaaaatgctc	240
published pksS	181	gacaatcgaattacaagagacgtaaaaaatgtgatgaaccaagaacaaatcaaaatgctc	240
pksS corrected	241	aacgtagtgaaagatatcgattttgtatccgatcatatgctggcaaaagacacacctgac	300
published pksS	241	aacgtagtgaaagatatcgattttgtatccgatcatatgctggcaaaagacacacctgac	300
pksS corrected	301	cataccgcctgagatcacttggtcatcaagcatttactccccgaaccattgaaaatctg	360
published pksS	301	cataccgcctgagatcacttggtcatcaagcatttactccccgaaccattgaaaatctg	360
pksS corrected	361	cgcggcagcattgaacaaattgctgaacagcttttagatgaaatggaaaaagaaaaataa	420
published pksS	361	cgcggcagcattgaacaaattgctgaacagcttttagatgaaatggaaaaagaaaaataa	420
pksS corrected	421	gcggatatcatgaaatccttcgcttccccctttgccttttattgttatatctgaattgatg	480
published pksS	421	gcggatatcatgaaatccttcgcttccccctttgccttttattgttatatctgaattgatg	480
pksS corrected	481	ggaatcccaaaagaagatcggtcacagtttcaaactctggaccaatgcgatgggtgatacc	540
published pksS	481	ggaatcccaaaagaagatcggtcacagtttcaaactctggaccaatgcgatgggtgatacc	540
pksS corrected	541	tctgaaggtaatagagagctgacaaatcaggcccttcgtgaatttaaagattatatcgct	600
published pksS	541	tctgaaggtaatagagagctgacaaatcaggcccttcgtgaatttaaagattatatcgct	600
pksS corrected	601	aagctgatccatgacagaagaataaaagccaaaagacgatttaacagcaaaacttgtgcat	660
published pksS	601	aagctgatccatgacagaagaataaaagccaaaagacgatttaacagcaaaacttgtgcat	660
pksS corrected	661	gctgaggaaaaacggcagcaagttaagcgaaaaagagctctattcgatgctgttcttgctc	720
published pksS	661	gctgaggaaaaacggcagcaagttaagcgaaaaagagctctattcgatgctgttcttgctc	720
pksS corrected	721	gttgtagccggccttgaaacaactgttaacttactcggctcaggcaccctcgattgctg	780
published pksS	721	gttgtagccggccttgaaacaactgttaacttactcggctcaggcaccctcgattgctg	780
pksS corrected	781	cagcacaagaaggaatgtgagaagctcaagcagcagcctgaaatgatcgctacagcggtt	840
published pksS	781	cagcacaagaaggaatgtgagaagctcaagcagcagcctgaaatgatcgctacagcggtt	840
pksS corrected	841	gaagaattgctgcgatacacctcacctgtcggttatgatggcaaatcggtgggccatcgaa	900
published pksS	841	gaagaattgctgcgatacacctcacctgtcggttatgatggcaaatcggtgggccatcgaa	900
pksS corrected	901	gactttacatataaggggcattcgatcaaaaaggagagacatgatttttataggcatcgga	960
published pksS	901	gactttacatataaggggcattcgatcaaaaaggagagacatgatttttataggcatcgga	960
pksS corrected	961	tctgccaatcgcgaccgcaatttttttgagaaccccgaaatattaataataaatcggtcg	1020
published pksS	961	tctgccaatcgcgaccgcaatttttttgagaaccccgaaatattaataataaatcggtcg	1020
pksS corrected	1021	cctaatagacatatcttttgggttttggcattcatttctgcttaggagcgccctcttgcc	1080
published pksS	1021	cctaatagacatatcttttgggttttggcattcatttctgcttaggagcgccctcttgcc	1080
pksS corrected	1081	aggctggaaggccacattgcatttaaagca-cttttgaagagatttctgatattgaact	1139
published pksS	1081	aggctggaaggccacattgcatttaaagcagcttttgaagagatttcc-----	1128
pksS corrected	1140	tgcggttgacactgatgacattcaatggagaaaaaatgtctttttaagaggattagaaag	1199
published pksS	1128	-----	1128
pksS corrected	1200	tctccctgtttcactttcaaaataa	1224
published pksS	1128	-----	1128

FIGURE II.9. Corrected amino acid sequence for experimental *pksS*

pksS corrected aa	1	MQMEKLMFHHPHGKEFHNNPFSVLGRFREEEPIHRFELKRF	40
pksS published aa	1	MQMEKLMFHHPHGKEFHNNPFSVLGRFREEEPIHRFELKRF	40
pksS corrected aa	41	GATYPAWLITRYDDCMAFLKDNRITRDVKNVMNQEQIKML	80
pksS published aa	41	GATYPAWLITRYDDCMAFLKDNRITRDVKNVMNQEQIKML	80
pksS corrected aa	81	NVSEDIDFVSDHMLAKDTPDHTRLRSLVHQAFTPRTIENL	120
pksS published aa	81	NVSEDIDFVSDHMLAKDTPDHTRLRSLVHQAFTPRTIENL	120
pksS corrected aa	121	RGSIEQIAEQLLDEMEKENKADIMKSFASPLPFIVISELM	160
pksS published aa	121	RGSIEQIAEQLLDEMEKENKADIMKSFASPLPFIVISELM	160
pksS corrected aa	161	GIPKEDRSQFQIWTNAMVDTSEGNRELTNQALREFKDYIA	200
pksS published aa	161	GIPKEDRSQFQIWTNAMVDTSEGNRELTNQALREFKDYIA	200
pksS corrected aa	201	KLIHDRRIKPKDDLISKLVHAEENGSKLSEKELYSMLFLL	240
pksS published aa	201	KLIHDRRIKPKDDLISKLVHAEENGSKLSEKELYSMLFLL	240
pksS corrected aa	241	VVAGLETTVNLLGSGTLALLQHKKECEKLKQQPEMIATAV	280
pksS published aa	241	VVAGLETTVNLLGSGTLALLQHKKECEKLKQQPEMIATAV	280
pksS corrected aa	281	EELLRYTSPVVMANRWAIEDFTYKGHSIKRGDMIFIGIG	320
pksS published aa	281	EELLRYTSPVVMANRWAIEDFTYKGHSIKRGDMIFIGIG	320
pksS corrected aa	321	SANRDPNFFENPEILNINRSPNRHISFGFGIHFCLGAPLA	360
pksS published aa	321	SANRDPNFFENPEILNINRSPNRHISFGFGIHFCLGAPLA	360
pksS corrected aa	361	RLEGHIAFKALLKRFPDIELAVAPDDIQWRKNVFLRGLES	400
pksS published aa	361	RLEGHIAFKAAFEIEIS-----	376
pksS corrected aa	401	LPVSLSK*	408
pksS published aa	376	-----	376

II.B.8. Using a new downstream primer, PKSS LONG DN SAA

A new downstream primer *pksS* long DN SAA, which would include the new stretch of sequence at the 3' end, was combined with the upstream primer *pksS* UP SAA for a PCR. The product was checked using gel electrophoresis, shown in figure II.10 and then purified using the "QIAquick PCR Purification Kit." After purification the gene underwent a restriction digest, shown in figure II.11, and ligation reaction with pQE60. Three different concentrations of the insert were used to ensure successful ligation into

the vector. Each ligation reaction was then transformed into competent M15[pREP4] cells and plated onto agar plates in preparation for sequencing. Transformants were screened for insert as before and one positive clone was sequenced as before. *According to the sequencing results, pksS was successfully cloned into the pQE60 plasmid, and all expected nucleotides minus the aberrant G1111, shown in Figure II.8, were present in the correct sequence.*

FIGURE II.10. Agarose gel of PCR products using pksS UP SAA and pksS long DN SAA

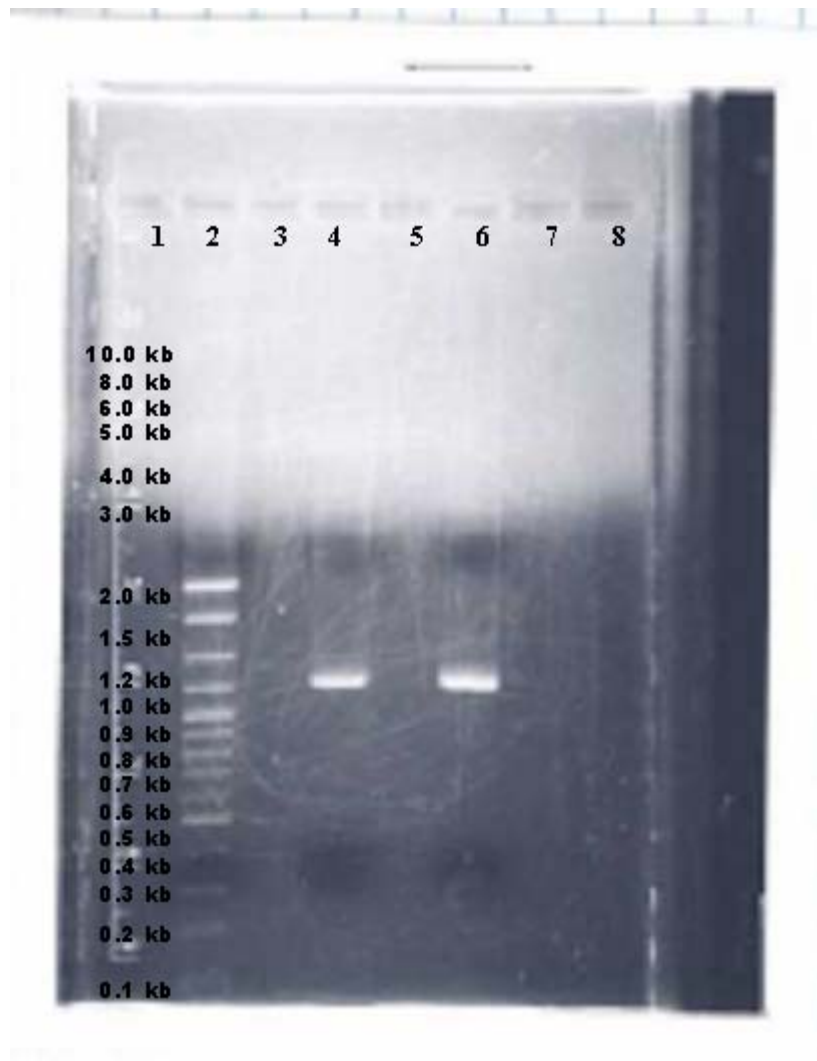
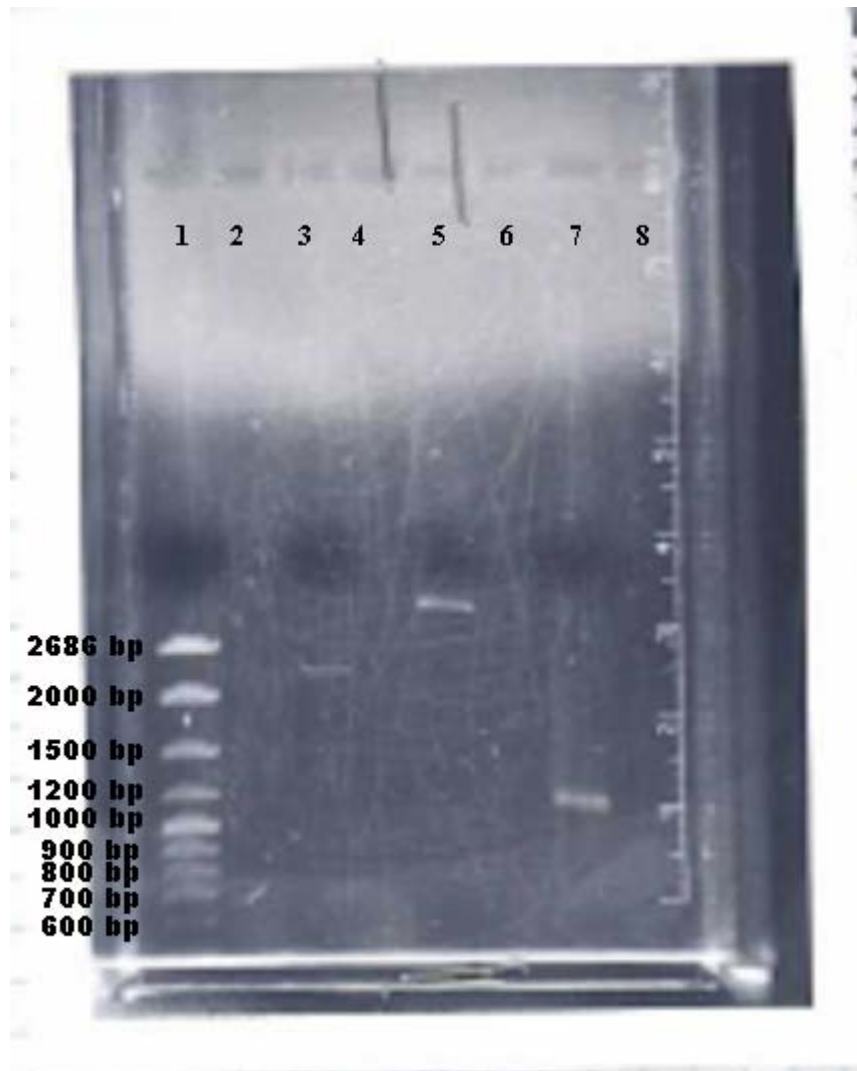


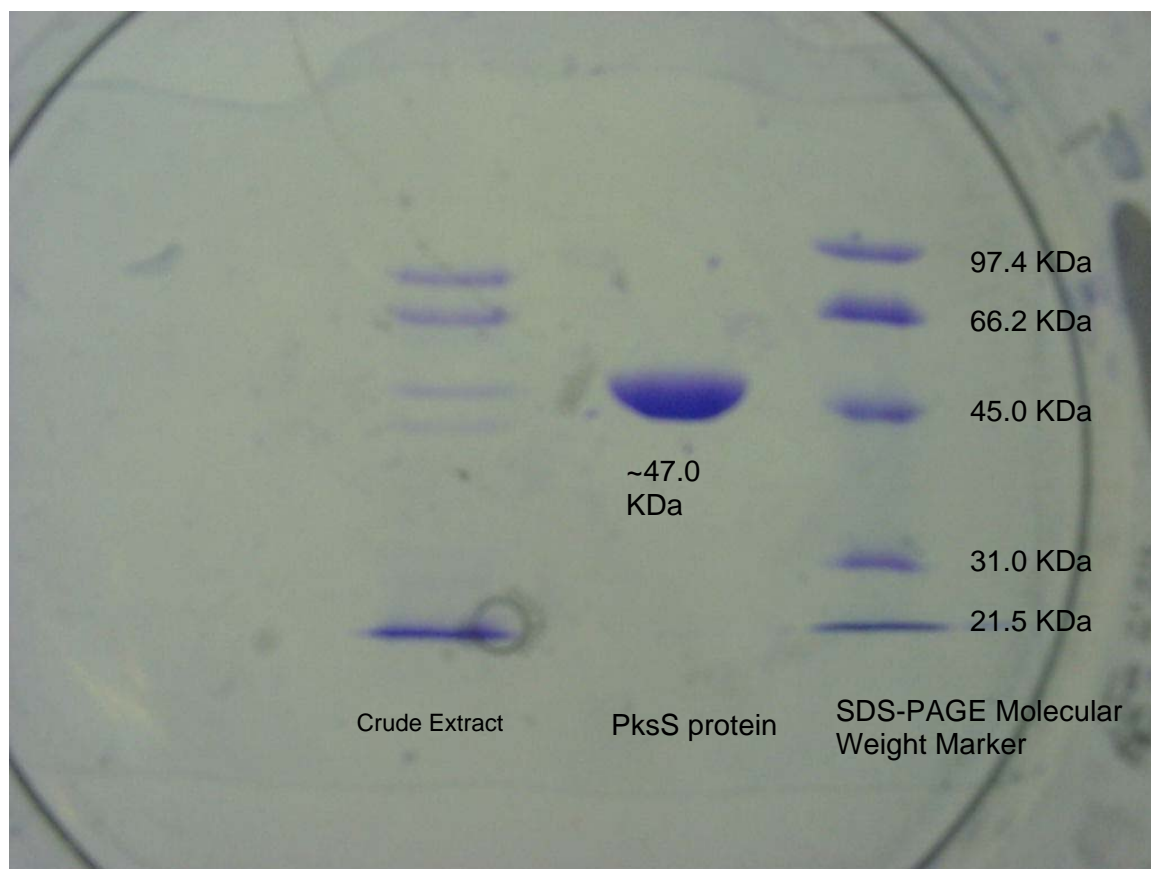
FIGURE II.11. Restriction digest of *pksS* and pQE60, Lane 1: Molecular Weight, Lane 3: Uncut pQE60, Lane 5: Cut pQE60, Lane 7: Cut *pksS*



II.B.9. Overexpression and purification of PksS

Following the successful cloning of *pksS* into pQE60, another transformant was used to inoculate a starter culture containing LB and dextrose. Following inoculation of the LB media, the cultures were induced with IPTG. The cells were then harvested and purified using a Ni-NTA column. Samples of the washes were collected and run through a SDS-PAGE gel. Figure II.12 shows the SDS-PAGE for the crude extract of *pksS* and the purified protein of *pksS*. A SDS-PAGE molecular marker was used to determine the approximate size of the purified *pksS*. Due to the 1 M imidazole within the elution buffer, which could interfere with subsequent spectroscopy, the protein was subjected to Sephadex G25 gel filtration to exchange the buffer. After the protein was added to the column, it was eluted using 50 mM potassium phosphate buffer at pH of 8.00.

FIGURE II.12. SDS-PAGE Gel of purified PksS



II.B.10 Sequence Homology

In an attempt to assess the importance of the newly annotated amino acid sequence in the structure of PksS, the corrected amino acid sequence was loaded into the Basic Local Alignment Sequencing Tool (BLAST), tool supplied by the National Center for Biotechnology Information. After performing “Protein-protein BLAST” with the sequences, the long list of similar candidates confirmed that PksS was likely a P450. The

sequence was aligned with the published sequence of PikC from *Streptomyces venezuelae* (Sherman 2006). The following 3D figure, obtained from the Swiss-modeling program, Deep View, shows a theoretical protein structure of PksS, built by homology with PikC. Figure II.13 shows that the additional sequence at the C-terminus is likely important to the overall protein structure. To highlight the importance of the additional sequence, figure II.14 was generated. The sequence that was not reported originally is colored lighter than the original sequence. A lack of this C-terminal sequence is expected to cause protein misfolding. With the completed structure, PksS is able to operate as a cytochrome P450. The heme has also been included in the structure to illustrate the location of the heme within the active site.

FIGURE II.13 PksS homology using PikC

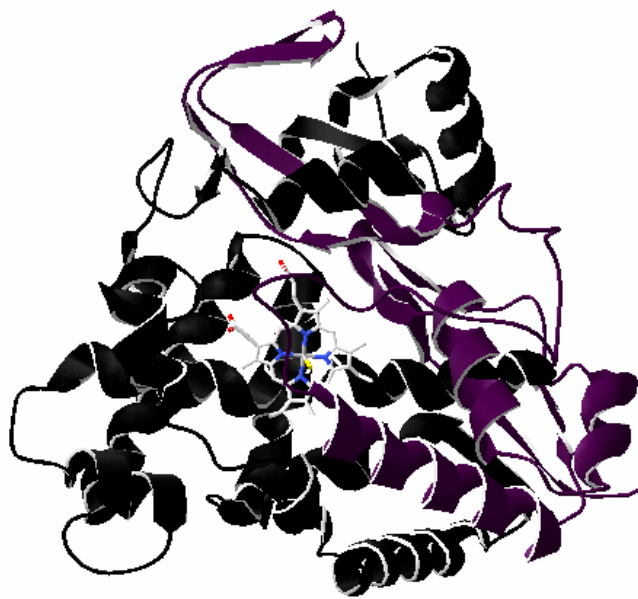
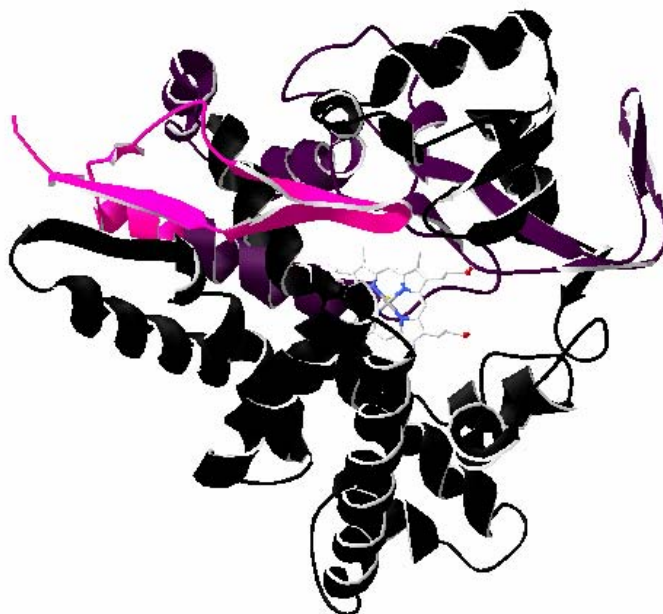


FIGURE II.14 Corrected sequence of PksS with unreported sequence highlighted in pink



II.C. Conclusion

After several attempts to clone *pksS* into pQE60 using the published sequence of *pksS* by the genomic database, it was determined there was an error within the sequence. Through several cloning and sequencing experiments, the error was identified as an artificial insertion within the reported sequence of *pksS* of a guanosine at the 1111th position. After correcting this error and searching the database for the next in-frame stop codon, *pksS* was determined to be 1224 base pairs long. Following this correction, primers were redesigned reflecting this frame shift and PCR was repeated. The product was then successfully ligated into the cloning vector using T4 ligase. This process

resulted in the successful cloning of *pksS* into the cloning vector pQE60. *pksS*/pQE60 was then overexpressed using the host cell M15[pREP4] in LB containing a 1% solution of dextrose. Following induction by IPTG, PksS was purified using a Ni-NTA column by utilizing the 6x His-Tag found at the C-terminus within the pQE60 cloning vector. The protein was eluted from the column elution buffer containing 1 M imidazole and then purified further using a Sephadex 25 column suspended in 50 mM potassium phosphate buffer, pH 8.0. The Sephadex column was used instead of dialysis for removal of the imidazole. Following the purification of PksS, the amino acid sequence was used to determine the function of PksS. Using homology to other cytochrome P450s, PksS was identified as a cytochrome P450.

II.D. Experimental

II.D.1 Cloning and overexpression of *pksS*

Initial attempts to clone *pksS* were unsuccessful because of primer errors. To increase the chances of successful amplification of *pksS*, two different upstream primers were designed with the following sequences, *pksS* UP1, 5' - TATCCCATGGAAATGGAAAAATTG - 3' (restriction site for NcoI) and *pksS* UP2, 5' - ATGCCCATGGAAAAATTGATG - 3' (restriction site for NcoI). The downstream primer, *pksS* DN 2, 5' - TATAGATCTGGAAATCTCTTCAAACG - 3' (restriction site for BglII) was used in both cases. The upstream primers were combined separately with the downstream primer, the genomic DNA template, purified from *Bacillus subtilis* 168, dNTP's, and Vent polymerase. The PCR was run at the following conditions: 94.0°C for two minutes; 30 cycles of 30 seconds at 98.0°C, 30 seconds at 60.0°C, and 90 seconds at

72.0°C; then a hold at 4.0°C. After PCR trials, it was determined that the primers were not annealing correctly to the template. At this point, new primers were designed, pksS pQEUP3, 5' - TATCCATGGAAATGGAAAAATTGATGTTTCATCCG - 3' (restriction site for *NcoI*) and pksS pQEUP4, 5' - ATGCCCATGGAAAAATTGATGTTTCATCCGCATGG - 3' (restriction site for *NcoI*).

Vent polymerase was utilized with the upstream primer, pksS UP4, under the following conditions: 94.0°C for two minutes; 30 cycles of 30 seconds at 98.0°C, 30 seconds at 50.0°C, and 90 seconds at 72.0°C; then a hold at 4.0°C. Due to differences in the sequences of the upstream primers, pksS UP3, was subjected to the following conditions: 94.0°C for two minutes; 30 cycles of 30 seconds at 98.0°C, 30 seconds at 45.0°C, and 90 seconds at 72.0°C; then a hold at 4.0°C. After the formation of the PCR product, the samples were purified using the QIAquick PCR Purification Kit. The PCR product and pQE60 plasmid were then digested separately with the restriction enzymes *NcoI* and *BglII* for 3.5 hours at 37.0°C. To inactivate the enzymes, the samples were then placed in a 65.0°C water bath for 20 minutes.

After incubation, a 0.7% low melting agarose gel was used to purify and measure the size of the material. The bands were excised from the gel and incubated in a water bath at 75°C. The products were then combined at three different concentrations described here: The insert:plasmid ratio for the 1st reaction was 2µL:1µL, for the second reaction 5µL:1µL, and for the 3rd reaction 1µL:2µL. Each reaction was mixed with water, Ligase buffer, and T4 DNA ligase. The samples were placed in a thermocycler overnight with the following conditions: 30 minutes at 37.0°C, 2 hours at 23.0°C, and then 4.0°C for

the remainder of the incubation. The following day, each mixture was transformed into competent DH5 α (*E. coli*) cells by combining 20 μ L of the ligation mixture to 100 μ L of the chemically competent cells. After a cold and heat shock, the cells were suspended in 1 ml of LB and incubated at 37.0°C for 1 hour. Following the incubation, the cells were plated on agar plates with a final concentration of 25 mg/ml of ampicillin and 30 mg/ml of kanamycin and incubated overnight at 37.0°C.

The DH5 α cells were made chemically competent by resuspending the cells in 3 ml of CaCl₂. After a 30 minute incubation, the cells were resuspended again in 1 ml of CaCl₂. Colonies from each reaction plate were used to inoculate starter cultures containing LB and 25 mg/ml of ampicillin and 30 mg/ml of kanamycin. The cultures were then incubated overnight at 37.0°C and purified using a QIAprep Spin Miniprep Kit in which the protocol Plasmid DNA Purification Using the QIAprep Spin Miniprep Kit and a Microcentrifuge was followed.

The plasmids were screened using PCR, in which *Taq* (HotMaster) polymerase was employed, and a restriction digest. Both reactions showed incorrect band sizes, therefore, the ligation was deemed unsuccessful.

II.D.2 Topo Cloning

Cloning was restarted from PCR using the primers and conditions as before. The polymerization resulted in an accurate sized band for the primers pksS UP3 and pksS DN 2 and an absence of bands for the primers pksS UP4 and pksS DN2. The process was repeated again for precautionary measures. The results were reproduced and it was determined that the upstream primer pksS UP3 was not annealing correctly.

Following the PCR, the product obtained for the upstream primer *pksS* UP 3 and the downstream primer *pksS* DN2 was purified using the “QIAquick PCR Purification Kit.” The next attempt to overexpress *pksS* into a new cloning vector, TOPO vector, required the “pBAD TOPO® TA Expression Kit.” Before the insert could be ligated into the vector, the product was combined with *Taq* polymerase, buffer, and dNTP's and incubated in the thermocycler at 72°C for 15 minutes. This incubation period provides necessary 3'-deoxyadenosine overhangs to the insert to allow for correct ligation into the TOPO vector.

The insert was ligated into the TOPO cloning vector by incubating the sample in a standard salt solution and vector for 15 minutes at room temperature. Following the incubation, the plasmids were transformed into “One Shot Top10 Competent Cells” by adding a small portion of a reaction into the competent cells. The mixture was incubated for 30 minutes on ice, heat shocked for 30 seconds, and then LB was added. The cells were shaken at 37°C for 1 hour. The cells were plated on agar plates containing 25 µg/ml of ampicillin and incubated overnight at 37.0°C. The following day, several starter cultures containing LB and 25 mg/ml of ampicillin were inoculated using colonies from the reaction plates. The starter cultures were purified using the “Plasmid DNA Purification Using the QIAprep Spin Miniprep Kit and a Microcentrifuge” to prepare the plasmid for screening. Gel electrophoresis showed that the cloning and ligation of *pksS* into the TOPO cloning vector was unsuccessful.

In another attempt to successfully clone the *pksS* gene, a new polymerase, Phusion High-Fidelity DNA Polymerase, was used. The PCR used the upstream primer

pksS UP3 and the downstream primer pksS DN2. The conditions were the following: a 30 second hold at 98.0°C; 30 cycles of 10 seconds at 98.0°C, 30 seconds at 59.0°C, and 30 seconds at 72.0°C; then a hold of 5 minutes at 72.0°C. Gel electrophoresis showed no bands, therefore, the cloning was unsuccessful.

II.D.3. Attempted cloning with new primers

The next attempt to clone *pksS* involved the fabrication of a new upstream primer, pksS UP SAA, 5' - CGAGGACCATGGAAATGGAAAAATTG - 3' (including a *NcoI* restriction site) and downstream primer, pksS DN SAA, 5' - CAAGTTCTAGATCTGGAAATCTCTTCAAAAGC - 3' (including a *BglII* restriction site). The PCR was conducted under the following conditions: a 30 second hold at 98.0°C; 30 cycles of 10 seconds at 98.0°C, 30 seconds at 59.0°C, and 30 seconds at 72.0°C; then a hold of 5 minutes at 72.0°C. Following PCR, the product was purified using the “QIAquick PCR Purification Kit.” The purified insert and pQE60 plasmid were digested for 3.5 hours at 37.0°C using the appropriate restriction enzymes. The enzymes were then heat inactivated by incubating the reactions in a 65.0°C water bath for 20 minutes. Following the incubation, the digested material was purified by 0.7% low melting agarose gel. Each band was removed from the gel and incubated in a water bath at 75°C to melt the agarose. The products were then combined at three different concentrations described here: The insert:plasmid ratio for the 1st reaction was 2µL:1µL, for the second reaction 5µL:1µL, and for the 3rd reaction 1µL:2µL. Each reaction was mixed with water, Ligase buffer, and T4 DNA ligase. The samples were placed in the

thermocycler overnight with the following conditions: 30 minutes at 37.0°C, 2 hours at 23.0°C, and then 4.0°C for the remainder of the incubation.

The following day, each mixture was transformed into competent M15[pREP4] cells by combining 20µL of the ligation mixture to 100µL of the chemically competent cells. After a 30 minute incubation on ice, the cells were heat shocked and then incubated at room temperature for 10 minutes. One ml of LB was added to the transformation mixture and then incubated at 37.0°C for 1 hour. Following the incubation, the cells were plated on agar plates with a final concentration of 25 µg/ml of ampicillin and 30 µg/ml of kanamycin and then incubated overnight at 37.0°C. Several starter cultures were inoculated using colonies from each concentration of the ligation mixtures and then purified using the “QIAprep Spin Miniprep Kit.” The product was screened for insert acceptance using a restriction digest. Gel electrophoresis show bands corresponding to the plasmid and insert. It was concluded that the cloning of *pksS* was successful.

Transformants from the previous reaction were then retransformed into host cell. A colony from each plate was used to inoculate starter cultures containing LB, 25 µg/ml of ampicillin, 30 µg/ml of kanamycin for the potential overexpression of PksS. Each starter was used to inoculate a Terrific Broth (TB) overexpression culture containing 2.4% Yeast, 1.2% Bacto Tryptone, 0.4% glycerol, 2.4% monobasic potassium phosphate, and 1.17% dibasic potassium phosphate. The TB also contained 25 µg/ml of ampicillin and 30 µg/ml of kanamycin. The overexpression culture was incubated for two days at 200 rpm at room temperature and then induced with 0.63 mM isopropyl-β-D-

thiogalactopyranoside (IPTG). After induction, the culture continued to grow at room temperature with a speed of 70 rpm for 24 hours. Following incubation, the cell cultures were centrifuged at 8,500 rpm at 4°C for 30 minutes. The pellet was stored at -80°C.

II.D.4. Attempted purification of PksS protein

The pellet from the previous step was resuspended in 20 ml of 1x Binding Buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9). The protein was lysed using a Sonic Dismembrator for 3 minutes and then centrifuging for 30 minutes at 11,500 rpm at 4°C. After syringe filtration, the protein was loaded on a 2 ml column bed of nickel-nitrilotriacetic acid (Ni-NTA) column. Following the adsorption, the column was washed using 20 ml of the 1x Binding Buffer. In the next wash, 12 ml of 1x Wash Buffer (60 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9) were used. The protein was eluted from the column using 12 ml of 1x Eluent Buffer (1 M imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9). During each wash, a 20 µL of the flow through was collected and combined with 80 µL SDS-PAGE buffer. The samples were run in a 12% SDS-PAGE to check if the protein had been extracted. No bands were seen in the gel.

II.D.5. Coexpression with pLysis RARE plasmid

After the unsuccessful attempt to overexpress PksS, a new vector was introduced to the overexpression system. To force *pksS* to express genes rarely used by *E. coli*, the Rosetta plasmid (pLys RARE) was transformed into M15[pREP4] along with pQE60/*pksS*. The transformation mixture was incubated on ice for 30 minutes, heat shocked, and then incubated at room temperature for 10 minutes. One mL of LB was added before the cells were incubated at 37.0°C for 1 hour. The cells were then plated on

agar plates containing 34 µg/ml of chloroamphenicol, 30 µg/ml of kanamycin, and 25 µg/ml of ampicillin and incubated overnight at 37°C. A colony was used to inoculate a starter culture that will be used for the overexpression of PksS. Following incubation, the starter culture was used to inoculate TB containing 34 µg/ml of chloroamphenicol, 30 µg/ml of kanamycin, and 25 µg/ml of ampicillin. The overexpression culture was incubated in a shaker at 240 rpm at 37 °C until the optical density at 595 nm reached 0.500-0.600. IPTG with a final concentration of 0.63 mM was added to the culture and incubation was continued for another 3 hours. Following the induction, 10 ml samples were removed periodically and centrifuged at 8,000 rpm. The cells were resuspended in 2 ml of 50 mM potassium phosphate buffer, pH 8.0 and then assayed using carbon monoxide and sodium dithionite to determine if a P450 was present, detailed in III.E.1. All peaks reported were inconsistent with the presence of a cytochrome P450. In combination with the assay, a 12% SDS-PAGE was used to determine if pksS had been expressed during the growth. Approximately 1 mL of cells were removed from the culture and centrifuged at top speed for 5 minutes. The cells were then resuspended in 1 mL of water to remove any contamination and then centrifuged again. The cells were resuspended in 80 µL of SDS-PAGE buffer. Using information collected throughout the growth period revealed that PksS protein had not been produced. For experimental purposes, the overexpression was repeated twice. In one case, the protocol was followed as before. In the second case, the concentration of IPTG was raised to 1.0 mM.

In another attempt to overexpress *pksS*, other transformants, which tested positive for *pksS* (detailed in II.E.3), were used to inoculate several starter cultures. Following

incubation, the starter cultures were used to inoculate Luria-Bertani (LB) cultures containing 0.5% yeast, 1.0% Bacto Tryptone, and 1.0% NaCl. The overexpression cultures were grown for several hours until optimal density was reached. At this point, they were induced with 0.63 mM IPTG and grown for three hours. Throughout the overexpression, 1 mL samples were removed every hour and centrifuged for 5 minutes at top speed. The cells were resuspended in 80µL of SDS-PAGE buffer and analyzed by SDS-PAGE. The gel showed a dim band around 46 KDa, the expected size of PksS. To increase the production of pksS, the media was prepared with 2% glycerol to enrich the culture. The LB was inoculated using starter cultures containing colonies from a previous transformation and grown according to standard protocol. Following incubation, 1mL samples were run through a SDS-PAGE. The gel showed no bands.

The next attempt to overexpress *pksS* utilized the introduction of a 1% dextrose solution to LB. Starter cultures were grown using colonies from the previous transformation containing 30 µg/ml of kanamycin and 25 µg/ml of ampicillin. The LB was grown until it reached optical density and then induced with 1 mM IPTG. Following induction, 1 mL samples were removed from the culture and run through a SDS-PAGE. Also, during the protein purification process, Ni-NTA column purification, samples (20 µL) were removed from each wash and analyzed by SDS-PAGE. The gels resulted in inconclusive information and the procedure was repeated again.

After several unsuccessful attempts to overexpressed pksS, colonies from the retransformation of pksS insert, pQE60, pRARE, and M15[pRep4] were used to inoculate starter culture 34 µg/ml chloramphenicol, 30 µg/ml of kanamycin, and 25 µg/ml

of ampicillin. The cultures were purified and then retransformed into competent M15[pREP4] cells. The transformation mixture was plated on agar plates containing 34 mg/ml of chloroamphenicol, 30 µg/ml of kanamycin, and 25 µg/ml of ampicillin. This transformation resulted in two different sizes of colonies, large and small. Both sizes of the colonies were used to inoculate starter cultures containing 34 µg/ml of chloroamphenicol, 30 µg/ml of kanamycin, and 25 µg/ml of ampicillin. Following their incubation, the starter cultures were used to inoculate LB containing 1% dextrose. The cultures were induced using 0.63 mM IPTG when optimal density was reached. During the growth period, several 1 mL samples were removed to determine if PksS was being produced using a SDS-PAGE. The results were inconclusive and the cultures were lysed and centrifuged in preparation for purification using a Ni-NTA column. During the purification, samples were removed from the washes and then analyzed by SDS-PAGE. The gel showed no conclusive evidence of PksS being expressed.

II.D.6. Sequencing

After several unsuccessful attempts to clone and purify pksS using plasmids such as pQE60 and pLys RARE, the next step was sequencing *pksS*. The insert was prepared for sequencing by transforming the insert and plasmid into competent M15[pREP4] cells. The colonies were grown in a starter cultures containing 30 µg/ml of kanamycin and 25 µg/ml of ampicillin, centrifuged, and then purified using the “QIAGEN QIAprep Miniprep Kit.” The concentration of DNA was determined by measuring the absorbance of the plasmid and insert at 260 nm. Upon receiving the results from the sequencing, it was

determined that our sequence lacked Guanosine 1111 reported for *pksS* from the genomic sequence for *Bacillus subtilis*.

II.D.7. Using new primers, PKSS UP SAA and PKSS DN2 SAA

After receiving the sequencing information, new primers were designed that would force the PCR to include G1111, if it was present. The upstream primer, pksS UP SAA, and the new downstream primer, pksS DN2 SAA, 5' - CAAGTTCTAGATCTGGAAATCTCTTCAAAAGC - 3' (including the restriction site *BglII*) were used in PCR with the following conditions: a 30 second hold at 98.0°C; 30 cycles of 10 seconds at 98.0°C, 30 seconds at 59.0°C, and 30 seconds at 72.0°C; then a hold of 5 minutes at 72.0°C. After polymerization, the product was purified and then digested in conjunction with pQE60 with the appropriate restriction enzymes for 3.5 hours. To inactivate the enzymes, the digested materials were incubated for 20 minutes in a 65.0°C water bath. The products were then purified using 0.7% low melting gel electrophoresis and extracted for ligation. The three different concentrations for the ligation reaction were: insert:plasmid ratio for the 1st reaction was 2µL:1µL, for the 2nd reaction 5µL:1µL, and for the 3rd reaction 10µL:1µL. Each reaction was combined with water, ligase buffer, and T4 ligase and incubated overnight under the following conditions: 30 minutes at 37.0°C, 2 hours at 23.0°C, and then held overnight at 16.0°C.

Following the incubation, the ligation mixtures were transformed into M15[pRep4]cells and plated on agar plates containing 30 µg/ml of kanamycin and 25 µg/ml of ampicillin. The colonies were used to inoculate several starter cultures containing the appropriate antibiotics. Each culture was purified and then digested using

NcoI and *BglII* restriction enzymes to verify the addition of the insert into the plasmid. After obtaining the results showing that the 10 µL concentration of the ligation mixtures yielded the desired products, the plasmids were retransformed into M15[pREP4]. For the purpose of sequencing and overexpression, the same colony was used to inoculate two starter cultures comprised of LB, dextrose, and 30 µg/ml of kanamycin and 25 µg/ml of ampicillin. Following the incubation, one starter culture was purified, concentrated, and sequenced. The second culture was used to inoculate an overexpression culture containing LB, dextrose, 30 mg/ml of kanamycin, and 25 mg/ml of ampicillin. The culture was grown to optimal density and induced with 1.0 mM IPTG. Subsequently, 1 ml samples were removed and treated with SDS-PAGE buffer. The samples were then analyzed by SDS-PAGE. The gel revealed that no pksS was being produced at any point.

Despite using primers that would force the PCR reaction to include G1111, sequencing still revealed an absence of G1111. After determining the error in the sequence, the genomic database was searched for the next in frame stop codon.

II.D.8. Using a new downstream primer, PKSS LONG DN SAA

After the sequence had been corrected, a new downstream primer pksS long DN SAA, 5' - GGGGCGTTTTGAAGATCTTTTTGAAAGTGAAACAGG - 3' (including restriction site *BglII*) was designed that reflected the lack of the guanosine 1111 and inclusion of the newly discovered portion of pksS. Another PCR was performed using the new downstream primer and the upstream primer pksS UP SAA with the following conditions: a 30 second hold at 98.0°C; 30 cycles of 10 seconds at 98.0°C, 30 seconds at 60.5°C, and 30 seconds at 72.0°C; then a hold of 5 minutes at 72.0°C. The product was

purified using the “QIAquick PCR Purification Kit” and then digested using the appropriate restriction enzymes for 3.5 hours at 37.0°C. After purifying the restriction digest using a 0.7% low melting gel, three different ratios of the insert:plasmid, 2µL:1µL, 5µL:1µL, and 10µL:1µL, were used in ligation reactions. Each ligation reaction was then transformed into competent M15[pREP4] cells and plated onto agar plates containing 30 µg/ml of kanamycin and 25 µg/ml of ampicillin. A colony from the 2µL:1µL concentration plate was used to inoculate a starter culture containing the appropriate antibiotics. Plasmids from each starter culture were then purified and concentrated. After being sequenced, it was determined that *pksS* was successfully cloned into the pQE60 plasmid, and all expected nucleotides were present in the correct sequence.

II.D.9 Overexpression and purification of PksS

Following the successful cloning of *pksS* into pQE60, another colony was removed from the 2µL:1µL ligation plate, and used to inoculate a culture containing LB, dextrose, 30 µg/ml of kanamycin and 25 µg/ml of ampicillin. Following the incubation, the starter culture was used to inoculate an overexpression culture containing LB, dextrose and the appropriate antibiotics. After reaching the optimum density of OD₅₉₅ 0.500-0.600, the culture was induced using 0.63 mM IPTG and grown for three hours. The culture was purified as before using a Ni-NTA column. Samples of the washes were collected and run through a SDS-PAGE. The gel showed that PksS had been purified and successfully eluted off of the Ni-NTA column.

Due to the 1 M imidazole within the elution buffer, which could interfere with subsequent spectroscopy, the protein was purified again using a Sephadex G25 column

with a diameter of 1.3 inches and a column bed of 5.3 inches. After the protein was added to the column, it was eluted using 50 mM potassium phosphate buffer at pH of 8.00. The Sephadex column was used rather than dialysis to purify the protein further.

II.D.10. Sequence Homology

To identify the role of PksS in the biosynthesis of bacillaene, sequence homology was used. The corrected DNA sequence was translated into the amino acid sequence and then used to search for any homologous sequences using the Basic Local Alignment Sequencing Tool (BLAST), a tool supplied by the National Center for Biotechnology Information. A homologous protein was found after performing “Protein-protein BLAST” search. The sequence was aligned with the published sequence of PikC From *Streptomyces venezuelae*. The sequence, identified by 2BVJ, was opened in the Swiss-modeling program, Deep View, to show the location of the heme relative to the active site.

CHAPTER III

CHARACTERIZATION OF PKSS

III.A. Introduction

After correcting the error within the published sequence of *pksS* and achieving a successful purification of the protein, the next step was to determine the function of PksS within the biosynthesis of bacillaene. Using sequence homology from II.E.9, PksS has preliminarily been identified as a cytochrome P450. The next step will be to classify PksS as a P450 through experimental methods. Once this is done, we will be able to determine the role of PksS in polyketide biosynthesis in *Bacillus subtilis*.

III.B. Goals

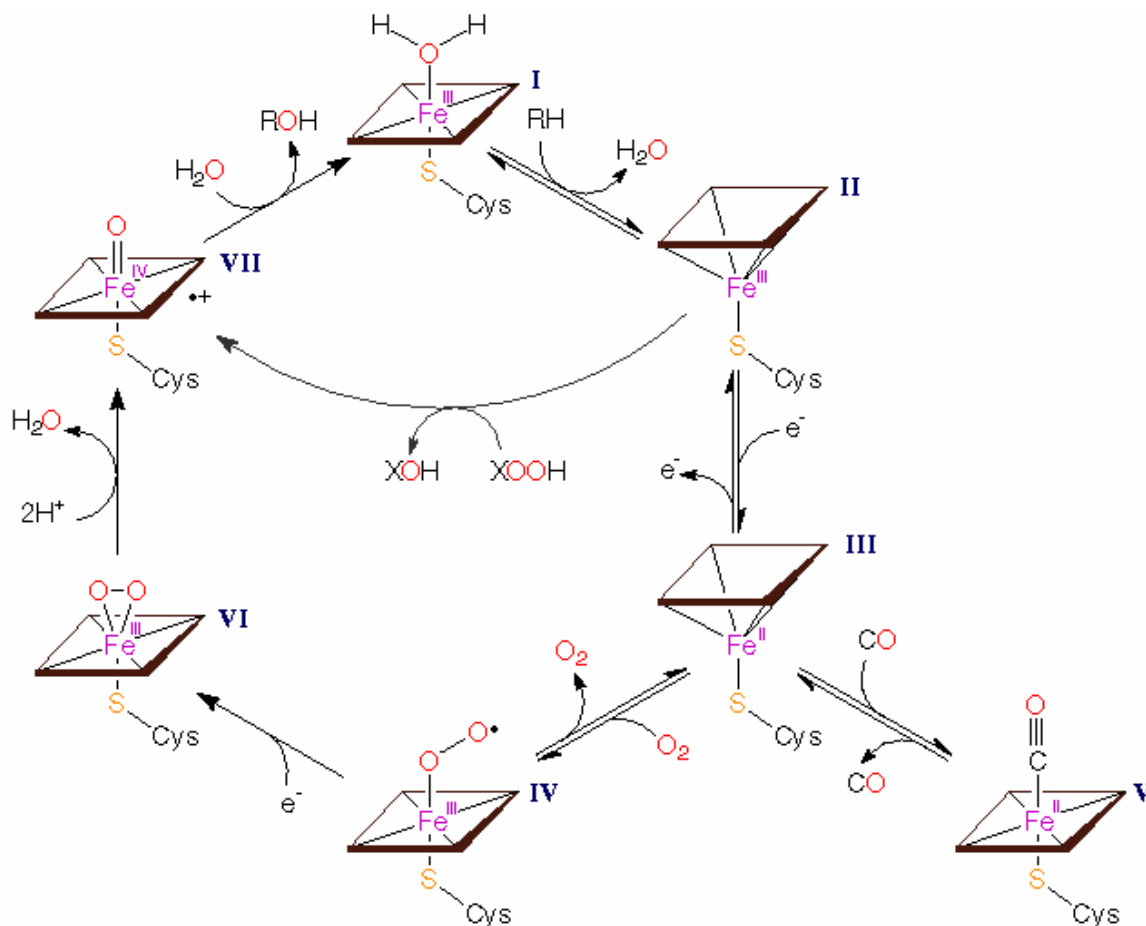
III.B.1. Identification of PksS as a P450

Sequence homology performed in II.E.9, has preliminarily identified *pksS* as a cytochrome P450. A major goal of this research was to classify *pksS* as a P450 through spectroscopy methods and characterize the function of PksS in the biosynthesis of bacillaene.

To determine if PksS is a cytochrome P450, the carbon-monoxide complex assay was used, shown in figure III.1. In this assay, PksS was combined with sodium dithionite, a reductant, and carbon monoxide. The assay is able to determine if PksS is a cytochrome P450 because the carbon monoxide acts as an inhibitor by blocking the heme center from regenerating through the use of molecular oxygen (Ortiz de Montellano 2005). The assay was performed by first reducing the iron center from a charge of III to

II by sodium dithionite, followed by the binding of carbon monoxide. This reduction and carbon monoxide binding is marked by a shift in wavelength from 420 nm to 450 nm, however, the shift does not remain at 450 nm. Because the carbon dioxide complex is a reversible inhibitor, molecular oxygen will bond to the Fe (II) center and generate a Fe (II)-oxy complex as the carbon dioxide is released. In the normal catalytic cycle of the enzyme, a second electron would be supplied to this complex by a reductase enzyme, generating the ferric peroxo-complex. The peroxo- intermediate is cleaved by protonation of the terminal oxygen releasing water and forming the iron IV-oxo intermediate. This species reacts with a substrate resulting in the hydroxylated product, which is then released and the cytochrome P450 is regenerated to its Fe (III) state (Ortiz de Montellano 2005). For complex III and VI to be formed from complex II and IV, two electrons must be donated. This is necessary for functional PksS, but we do not know what provides them in *Bacillus subtilis*.

FIGURE III.1. Mechanism of Carbon Monoxide Inhibition of PksS (Andersson 1991)



All P450 enzymes have a characteristic visible absorption spectrum when reduced in the presence of carbon monoxide. This inhibition is achieved through the addition of carbon monoxide. The carbon monoxide covalently binds to the iron center, where the carbon donates electrons to the iron center through the sigma bond (Ortiz de Montellano 2005). During this donation, there is also a back-donation of electrons from the Fe (II) d -orbitals to the unoccupied π^* -orbital of the ligands. This donation of electrons, leads to the reversible binding to Fe III. This binding allows for the monitoring of the reduced

carbon monoxide complex of the iron center through a wavelength shift from 420 nm to 450 nm (Ortiz de Montellano 2005).

Following the determination of PksS as a cytochrome P450, strains of *Bacillus subtilis* will be screened for reduction capabilities. Two strains in particular, *Bacillus subtilis* 168 and OKB105 will be used because of their genetic ability to produce bacillaene. It is expected that PksS will become reduced when combined with an electron donor, such as NADPH and NADH, and the crude protein extracts. This reduction of the Fe can be monitored by spectroscopy when carbon monoxide shifts the Soret peak from 420 nm to 450 nm.

By performing the reductase experiment, two goals can be reached. The first, more information of the function of PksS in the polyketide biosynthesis of bacillaene will be available. Several hypotheses have been described earlier detailing the hypothetical role of PksS in polyketide metabolisms. Through liquid chromatography mass spectrometry (LC-MS), it may be possible to determine whether bacillaene, dihydrobacillaene, or other compounds are the substrate for PksS.

There are two types of reductases for the cytochrome P450 enzyme. In Class I, the electrons are transported through a two-step shuttle in which ferredoxin (an iron-sulfur complex) and a ferredoxin reductase is used. This class is most commonly associated with mitochondria and bacterial P450s and can be identified by the use of NADH as the electron donor (Ortiz de Montellano 2005). The second class, Class II, shuttles electrons through a single-membrane bound enzyme, NADPH reductase. This

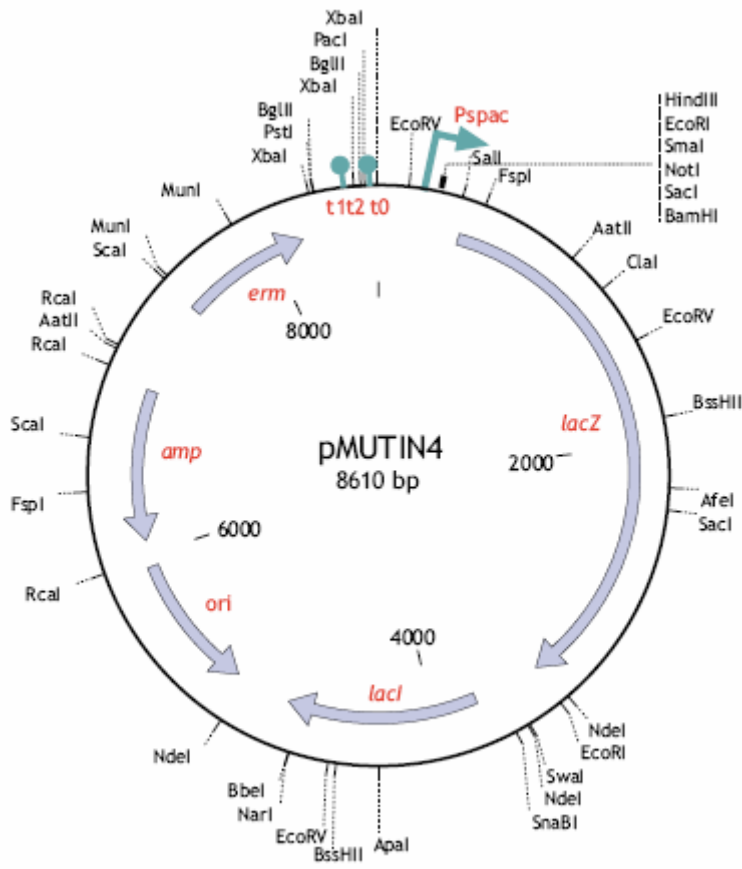
class is associated with microsomal P450s and utilizes diflavin reductases, containing FAD and FMN (Ortiz de Montellano 2005).

III.B.2. Constructing *pksS* mutants

In attempts to characterize the role of PksS, a mutant of *pksS* will be constructed using *Bacillus subtilis* 168 as the genomic template. In theory, disrupting *pksS* will ultimately lead to the disruption of the final product in the polyketide synthesis (Vagner 1998). Monitoring the products compared to the original extracts with liquid chromatography mass spectrometry may lead to some insight on the role of PksS in polyketide biosynthesis.

For knockout mutation of *pksS*, the cloning vector pMUTIN4, shown in figure III.2 will be used. This vector has been designed specifically for the inactivation of specific genes from *Bacillus subtilis*. The pMUTIN plasmids have incorporated different characteristics into the vector that allow for the deactivation of *Bacillus subtilis* genes (Vagner 1998). Along with gene inactivation, the plasmids contain the Pspac promoter, which controls the transcription of downstream genes through IPTG. By controlling the downstream genes, the transcription and time induction of the mutated gene can be controlled. The Pspac promoter also incorporates a modified *lacI* gene that can be induced within *B. subtilis* (Vagner 1998).

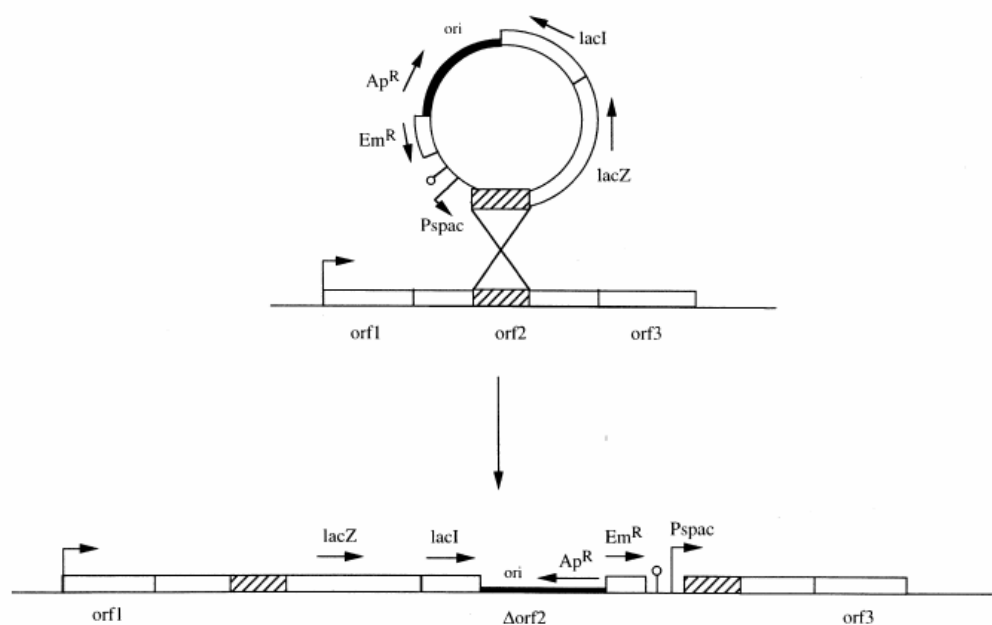
FIGURE III.2. pMUTIN cloning vector (Vagner 1998)



pMUTIN4 cannot replicate within *B. subtilis*, however, it can replicate in *E. coli* using the ColE1 origin site. This plasmid contains the β -lactamase gene, which allows ampicillin selection in *E. coli*. This is important because *E. coli* can be used as the host to assemble the plasmid that will target *pksS*. Since the plasmid lacks a *Bacillus subtilis* origin of replication, the only way the *B. subtilis* can survive erythromycin selection is through homologous recombination of the plasmid into the genome (Vagner 1998).

The knockout mutation is achieved by a crossover reaction, shown in figure III.3. The crossover of the vector into the genome disrupts the targeted gene and also incorporates antibiotic resistance and the *lacZ* and *lacI* genes (Vagner 1998). Using PCR and restriction digests, several screens will be performed to ensure that the mutation of *pksS* was effective.

FIGURE III.3. Crossover reaction in pMUTIN4 (Vagner 1998)



The Vagner group, which developed pMUTIN, found that the mutation process was optimal for an insert length between 250-300 bp. We will therefore, amplify a ~300 bp sequence from the middle of *pksS* using Taq polymerase and PCR. Following the amplification, the insert will be ligated into pMUTIN4, via the multiple cloning site

(MCS). The plasmid will then be transformed into *Bacillus subtilis*, resulting in a knockout mutation of *pksS*.

III.C. Results

III.C.1. P450 Assay

The first step in characterizing PksS as a cytochrome P450 required the use of a P450 assay. To perform this assay, cells from an overexpression culture were collected after induction with IPTG and resuspended in potassium phosphate, carbon monoxide and sodium dithionite (Phillips 1998). If PksS is a P450, this treatment is expected to result in a shift in a Soret peak from 420 nm to 450 nm. Using whole cells in this way demonstrated that PksS is producing cytochrome P450. We confirmed this result with purified protein.

To ensure that the PksS is the origin of a 450 nm Soret peak, protein obtained during the purification using a Ni-NTA column was collected and assayed. Several controls were also run as a comparison for the data obtained. The controls used were: PksS in 50 mM potassium phosphate buffer, pH 8.0; PksS, carbon monoxide, and 50 mM potassium phosphate, pH 8.0; and PksS, sodium dithionite, and 50 mM potassium phosphate. The full reaction contained PksS, carbon monoxide, sodium dithionite, and 50 mM potassium phosphate. The following figures, figure III.4, III.5, III.6, and III.7, represent the data obtained from the purified protein from PksS.

Figure III.4. PksS in Potassium Phosphate Buffer, 50 mM

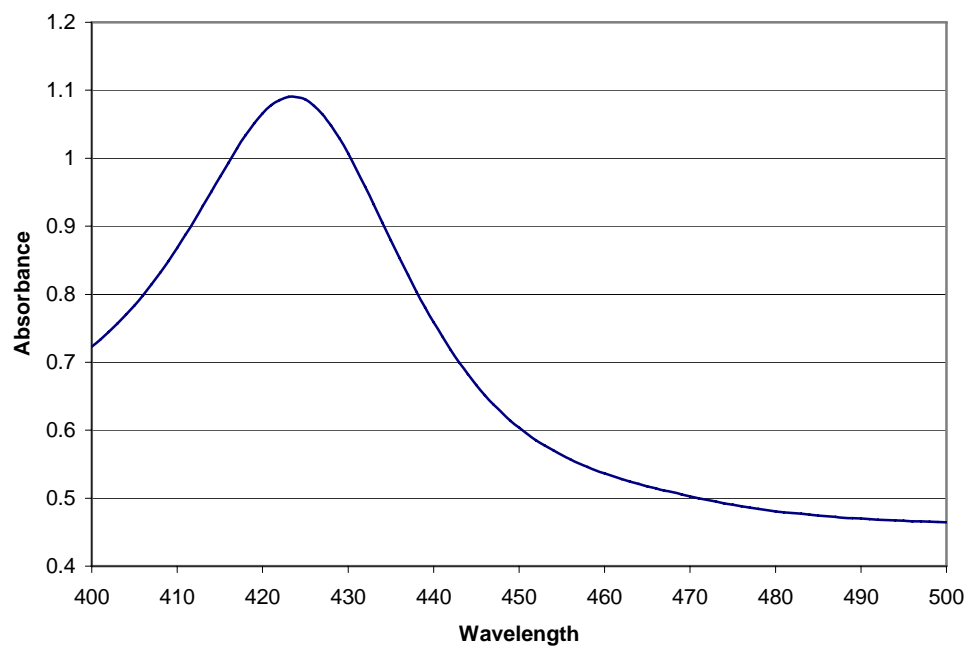


Figure III.5. PksS with carbon monoxide in potassium phosphate buffer, 50mM

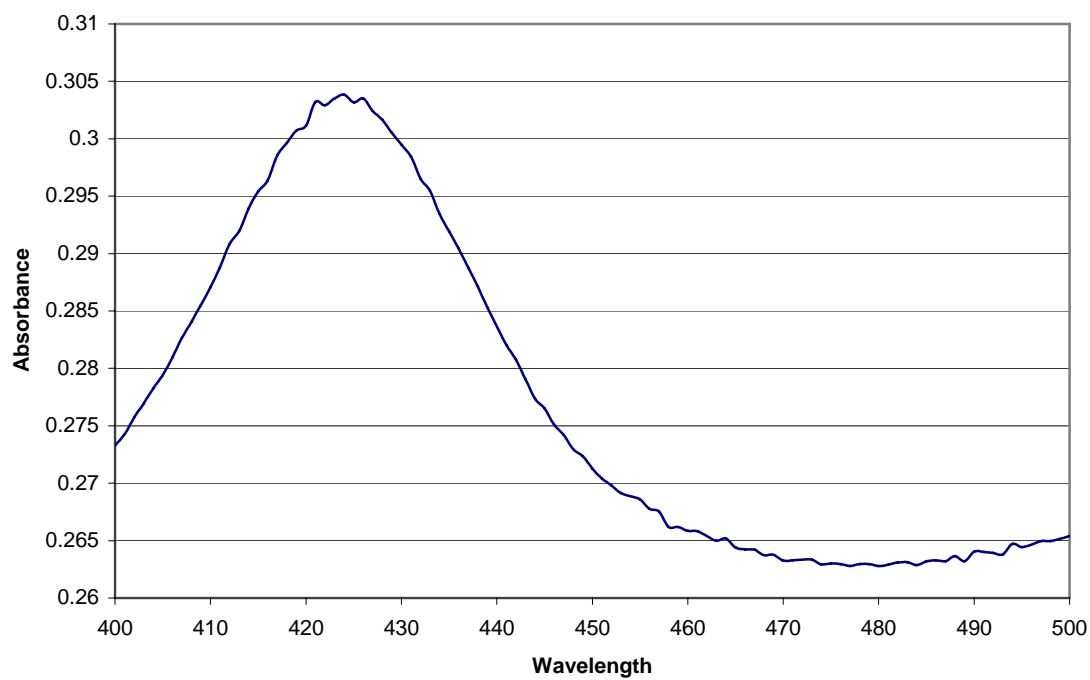


Figure III.6. PksS with dithionite in potassium phosphate buffer, 50mM

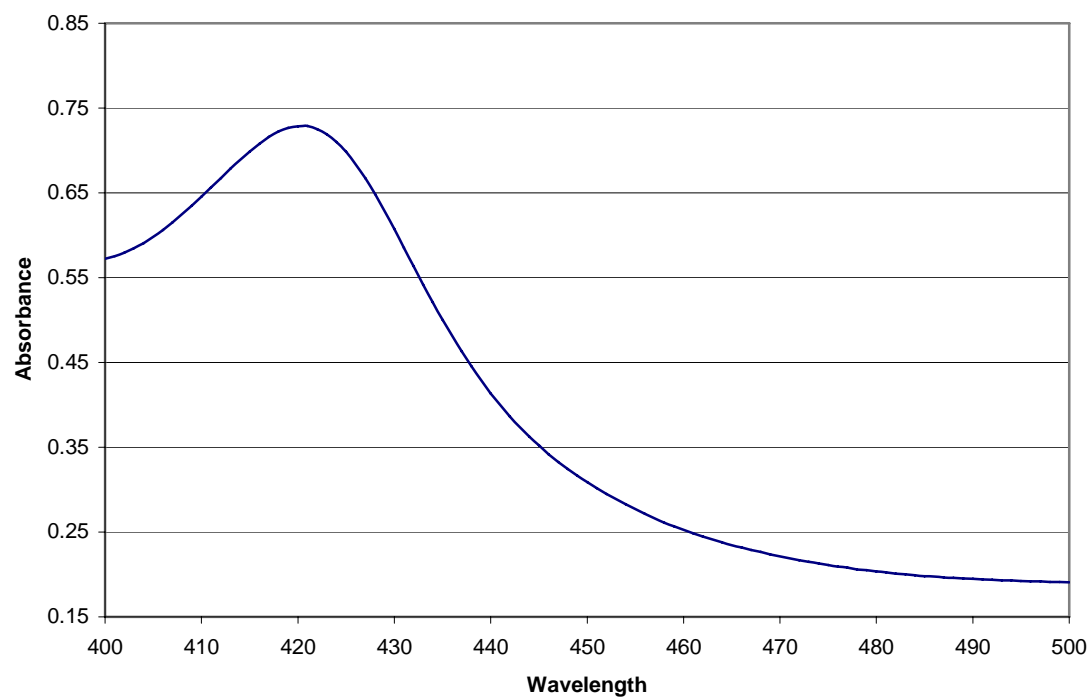


Figure III.7. Full P450 reaction, containing PksS, CO, dithionite, and potassium phosphate Buffer, 50mM

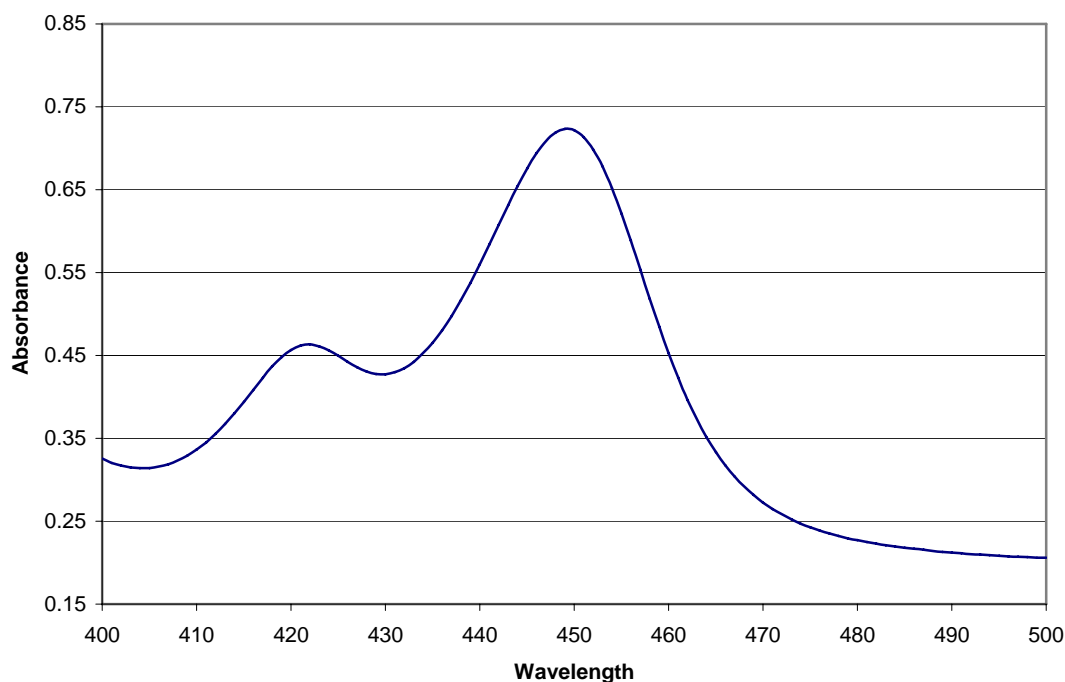


Figure III.7 shows that when PksS is combined with sodium dithionite (a reductant) and carbon monoxide, the heme center resulted in a wavelength shift from 420 nm to 450 nm, which is characteristic of the heme-Fe⁺² CO complex.

III.C.2. Establishing a suitable P450 reductase system for PksS

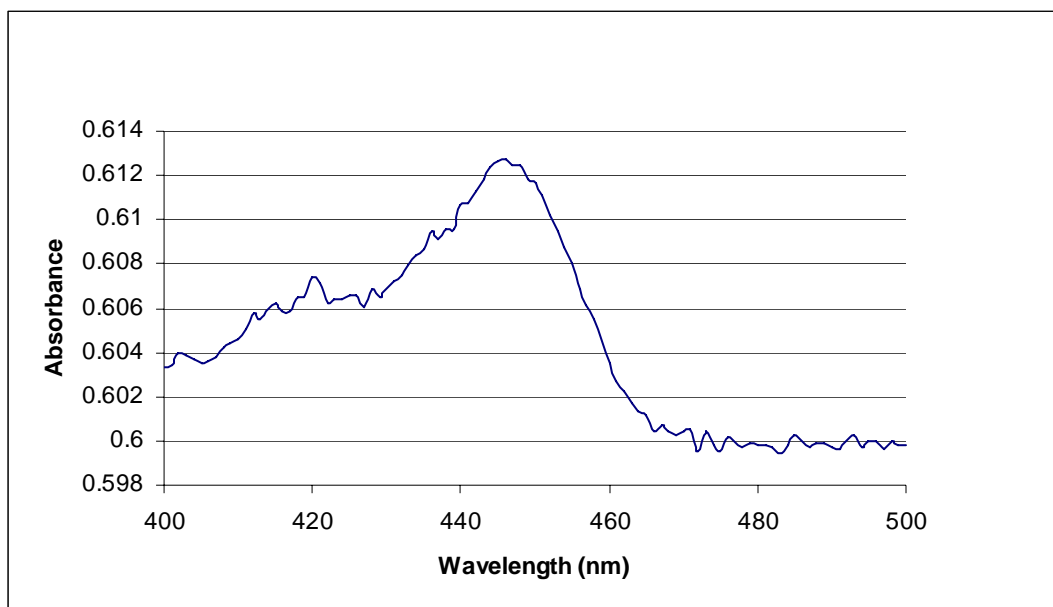
After classifying PksS as a cytochrome P450, two *Bacillus subtilis* strains were screened for P450 reductase activities. Two specific strains have been identified as possible candidates for reducing PksS. Both BS168 and OKB105, were chosen because they both have the *pksX* cluster. After growing both strands in Landy media (a minimal

media) and LB media (a rich media), LB was determined to be the most effective growing media. After growing both strains under aerobic conditions, the crude protein was extracted and stored for later use in experiments.

In assays for PksS reductase activity, each reaction was carried out under anaerobic conditions. Any access to molecular oxygen will inhibit the iron center from being reduced when combined with carbon monoxide. Rather than the P450, if any molecular oxygen is present, the reductase will reduce the molecular oxygen with NADH or NADPH. In combination of protein extracts from the two different strains of *Bacillus subtilis*, different electron donors, NADH (Class I) and NADPH (Class II), were used for the reduction of PksS (Ortiz de Montellano 2005).

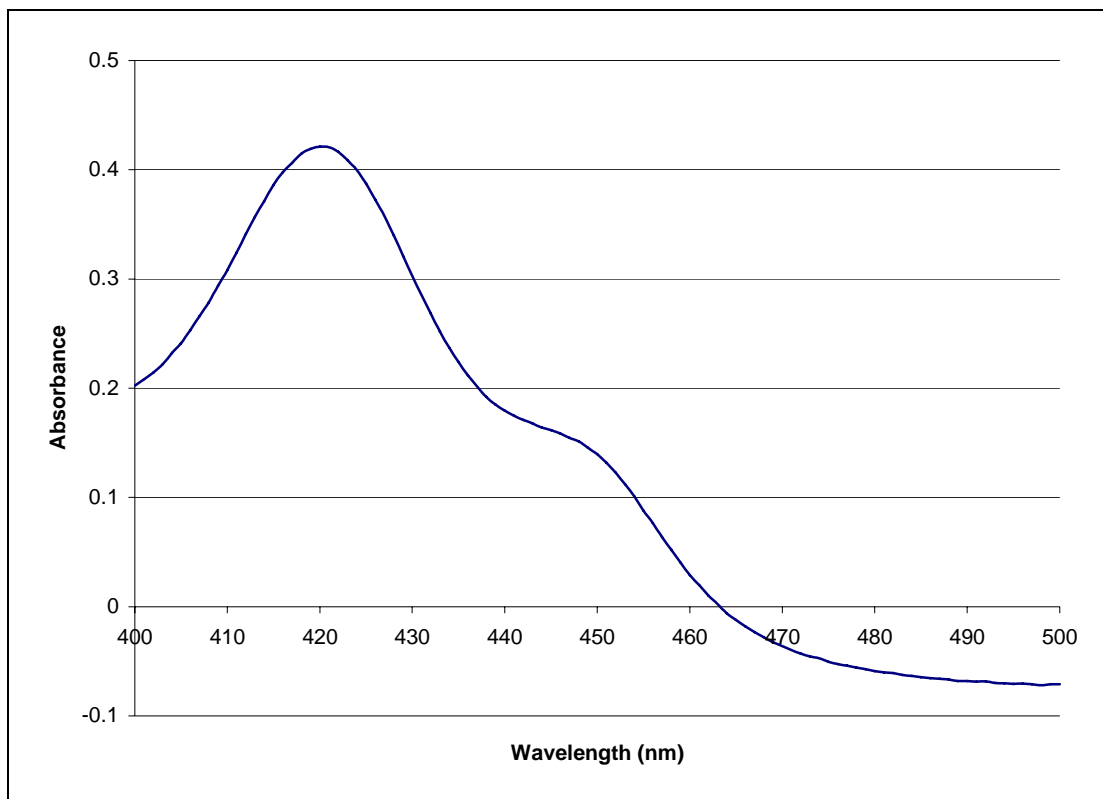
To find a suitable reductase, PksS was combined with 50 mM potassium phosphate, pH 8.0, and NADH or NADPH. After purging the cell with argon gas to maintain anaerobic conditions, the cell was further purged with carbon monoxide. Following the addition of crude extract from either BS168 or OKB105, PksS was expected to reduce to Fe II and form the carbon monoxide complex, shown through a shift in wavelength from 420 nm to 450 nm. To ensure that we had conditions sufficient for measuring the reductase activity, a known cytochrome P450_{BM3}, donated by Dr. Gregory Raner, was used as a positive control. BM3 was used because it is a cytochrome P450, which contains a reductase fusion domain. The demonstration of BM3 self-reduction would indicate that our experimental conditions are sufficient for the assay of reductase activity with an uncharacterized system. A spectrum recorded for this positive control with BM3 is shown in figure III.8.

FIGURE III.8. BM3 control for reductase activity



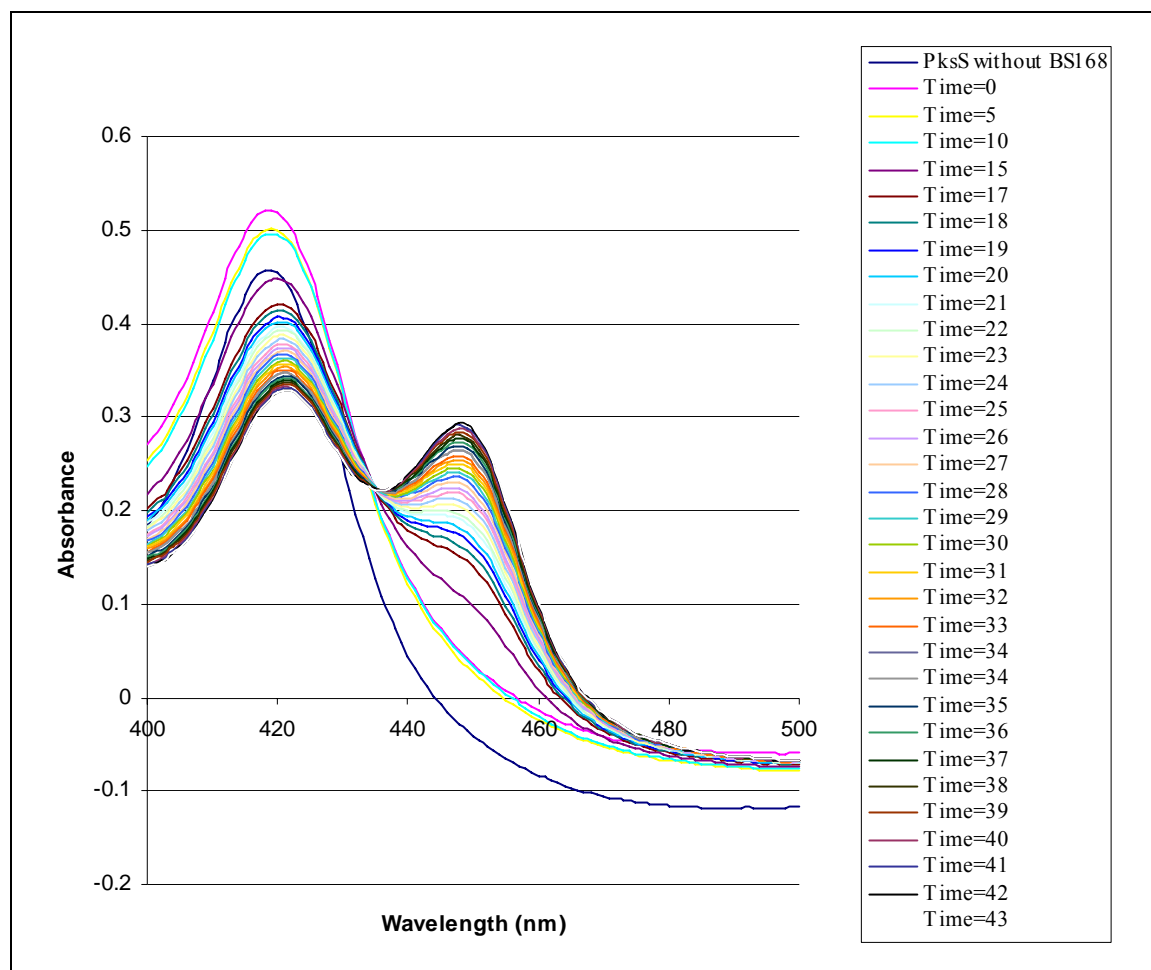
The observation of a reduced carbon monoxide spectrum for BM3 provided insight that the conditions for generating this species were successfully met. After determining that our reductase activity was achieved successfully with this positive control, we then turned to tests for PksS activity. The first sample contained PksS and NADH in 50 mM potassium phosphate pH 8.0. After purging the sample with argon gas and evacuating the system under high vacuum, the sample was purged with carbon monoxide for several minutes. The protein-extracts, which presumably contained the reductase, were introduced to the PksS sample using a syringe needle. The visible spectrum was taken, shown in Figure III.9. The spectrum shows that BS168 protein extracts and NADH can reduce the heme within PksS.

FIGURE III.9. BS168, NADH, and PksS in 50 mM potassium phosphate, pH 8.0



Spectra were continually collected for 42 minutes to show the time-dependence of the PksS reduction. Figure III.10 shows the peak at 450 nm continues to increase as the peak at 420 nm decreases. The shift in wavelength represents the iron center being reduced from Fe (III) to Fe (II) with carbon monoxide binding to the Fe (II) state.

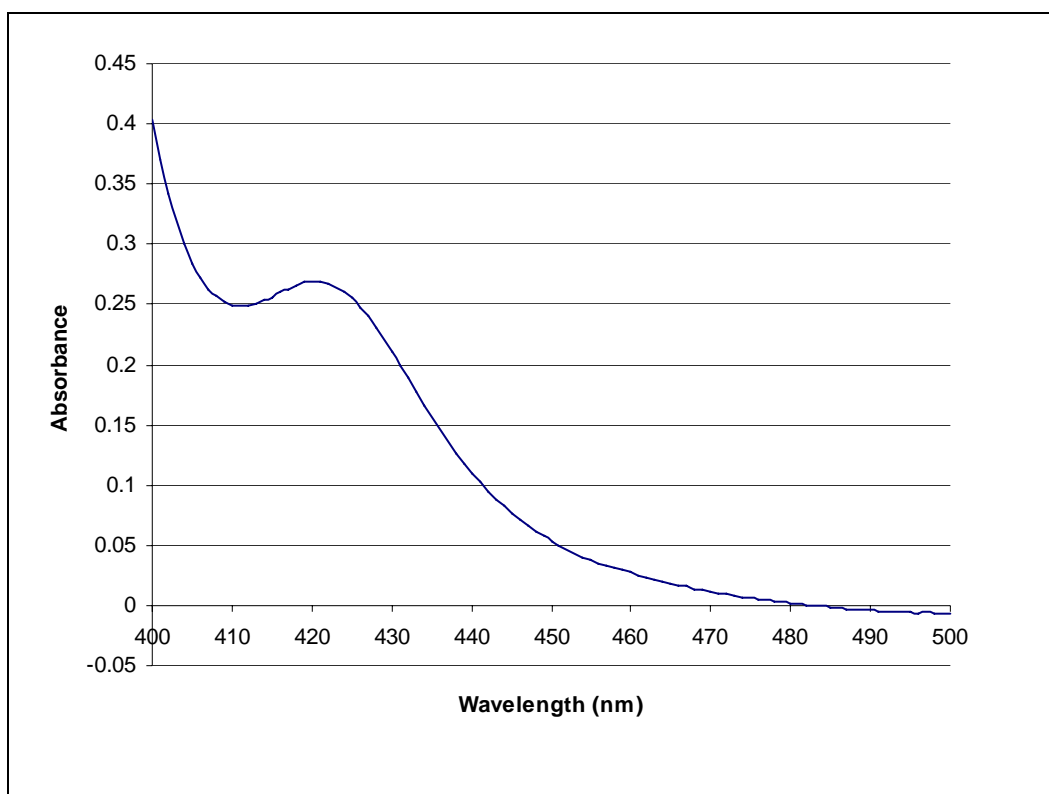
FIGURE III.10. Complete reduction of PksS using *Bacillus subtilis* 168 crude extracts



The next combination that was analyzed was PksS and NADPH in 50 mM potassium phosphate pH 8.0. The sample was purged with argon gas, evacuated, and purged again using carbon monoxide as before. After the sample was prepared, BS168 was added to the sample using a syringe needle and the visible absorption spectrum was taken, shown in figure III.11. The sample was allowed to incubate for more than one-

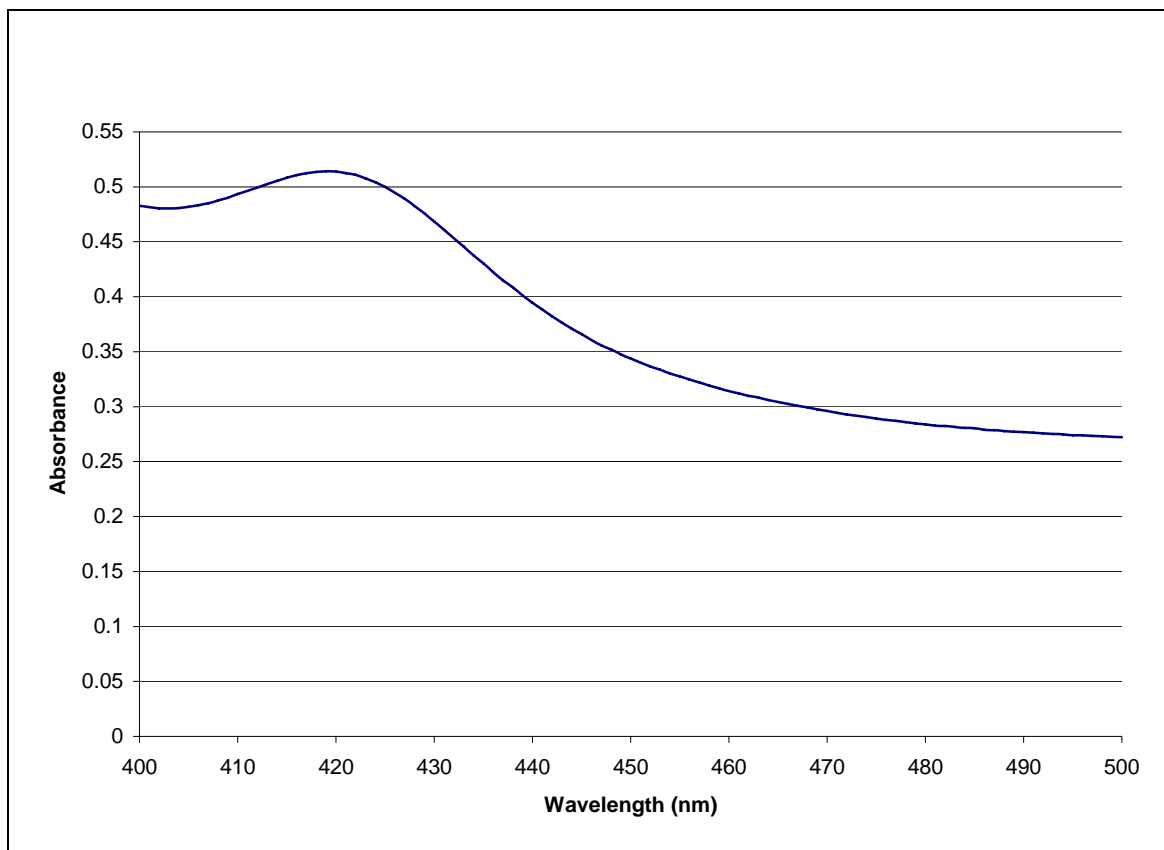
hour and scanned continuously to check for any reduction. The spectrum shows that with the addition of NADPH, PksS was not reduced.

FIGURE III.11. BS168, PksS, and NADPH in 50 mM potassium phosphate, pH 8.0



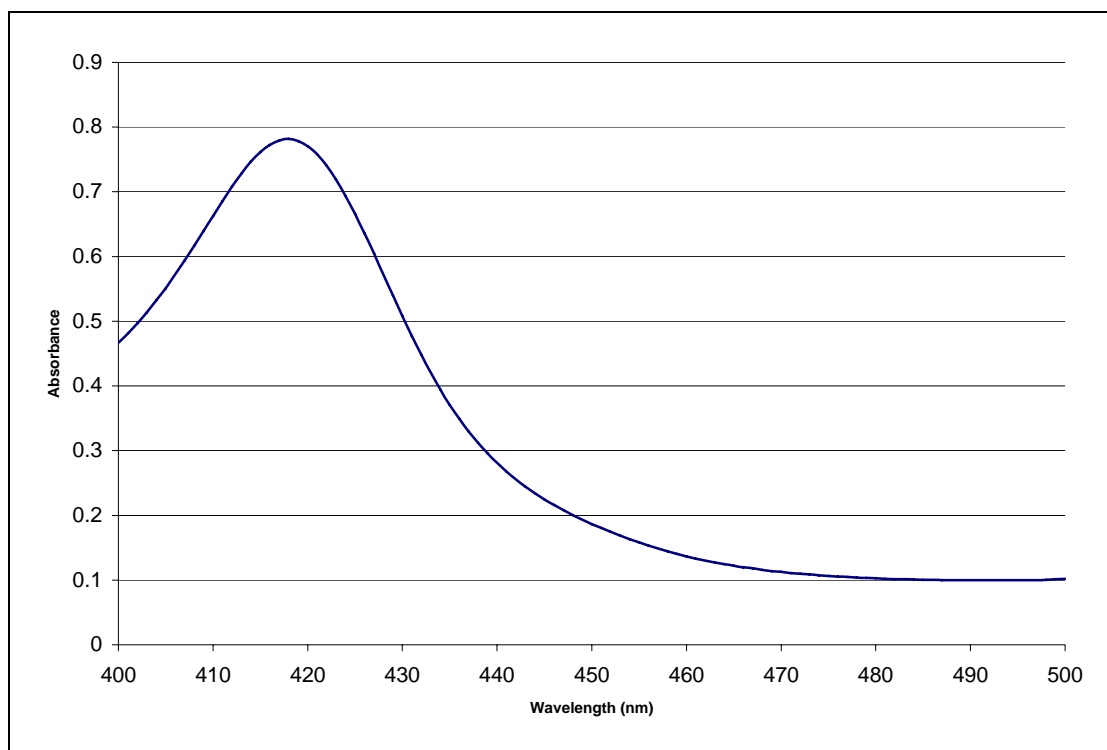
The third sample was prepared with PksS and NADH using the same procedure described previously. After the sample was purged with carbon monoxide, OKB105 was added to the cuvette using a syringe needle. The sample was then scanned for reductase activity, shown in figure III.12. The spectra show that with the addition of NADH and OKB105 crude extract, PksS is not reduced into its P450 form. Therefore, the combination of NADH and OKB105 do not have the capability to reduce PksS.

FIGURE III.12. OKB105, PksS, and NADPH in 50 mM potassium phosphate, pH 8.0



The final sample was prepared by combining PksS and NADPH in a sealed cuvet. After purging the sample with argon gas and evacuating the system under high vacuum, the sample was then purged with carbon monoxide for several minutes. Crude extract from OKB105 was injected into the cuvette using a syringe needle. The sample was then scanned for any reductase activity, shown in figure III.13. The absence of a peak at 450 nm shows that the combination of OKB105 and NADPH was not effective in the reduction of PksS.

FIGURE III.13. OKB105, NADH, and PksS, in 50 mM potassium phosphate, pH 8.0

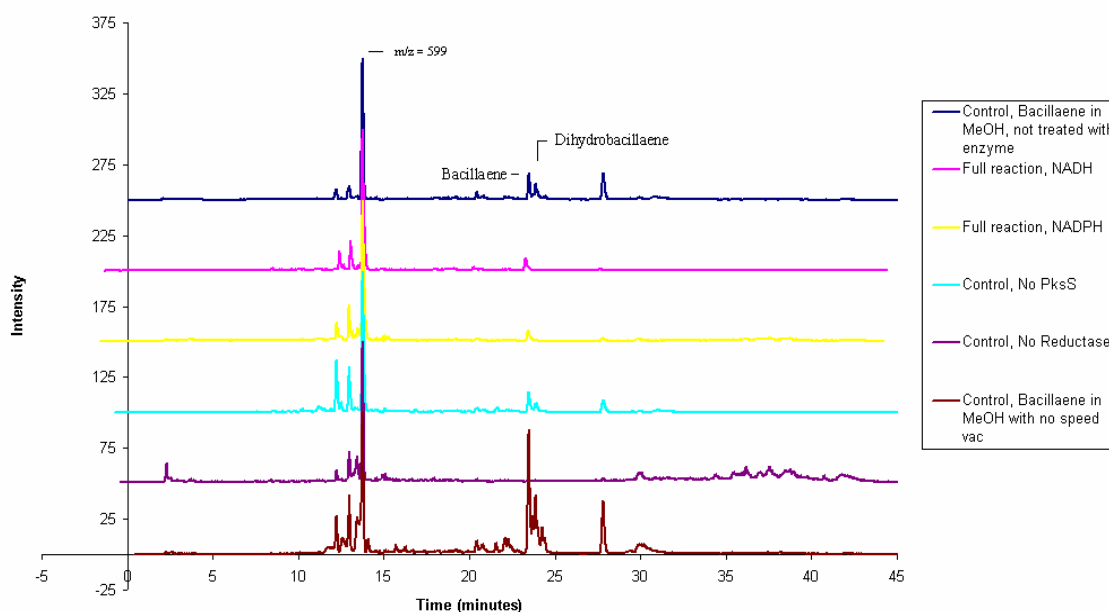


III.C.3. Determining the role of PksS using LCMS

After establishing a P450 reductase for PksS, LCMS was used to identify the role of PksS in the biosynthesis of bacillaene. The first step of preparing the samples for LCMS required a large amount of PksS to be obtained. After the protein was purified using both the Ni-NTA column and the Sepharose G25 column, the protein was kept cool until the procedure began. Several cultures of BS39374, a strain of *Bacillus subtilis*, were prepared by growing in Landy media for two days. After acquiring crude bacillaene through methanol extractions, the samples were combined with the crude PksS reductase, an electron donor, and PksS. The samples were analyzed by LC-MS over a period of 45

minutes in which the organic mobile phase increased from 5% to 95%. Some of the data obtained show peaks relating to the production of bacillaene and hydrobacillaene. The chromatograms for the reactions listed in Table III.1 are shown below. All chromatograms have been offset for clarity and to diminish the effect of drifting by the HPLC.

FIGURE III.14. Methanol extraction of bacillaene



The chromatograms show a major peak of 599 m/z, which is consistent with either hydrated bacillaene or hydroxylated dihydrobacillaene. The other two less intense peaks, with 581 and 583 m/z, correspond to bacillaene and dihydrobacillaene, which has not been previously observed. It is important to note that during the full reactions in which PksS and crude reductase was added to the sample, the peaks corresponding to

dihydrobacillaene are no longer present. These results, although not conclusive, suggest that dihydrobacillaene may be the substrate for PksS. In order to positively identify the role of PksS in polyketide synthesis, knockout mutations of PksS were designed to determine the possible product of this P450. We expect to see a change in m/z in the chromatogram to determine the effect of PksS on bacillaene.

III.C.4. Construction of *pksS* Mutant

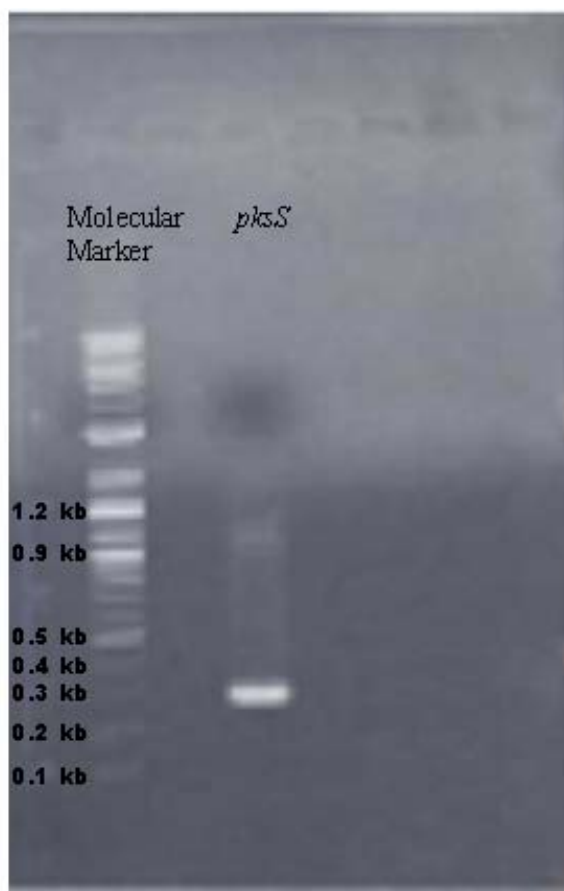
After being unable to positively identify the full role of PksS in the polyketide synthesis of bacillaene by HPLC, mutants of *pksS* were generated using the cloning vector pMUTIN4. After selecting a specific area of the gene to clone into pMUTIN4, shown in figure III.15, primers were designed to amplify this portion of the gene.

FIGURE III.15. Sequence alignment of corrected *pksS* highlighting the region used to clone into pMUTIN4.

pksS corrected	1	atgcaaatggaaaaattgatgtttcatccgcacggttaaagagtttcatcacaatcctttt	60
pksS mutant	1	-----	1
pksS corrected	61	tcagtttttaggacgatttagagaggaagagcccattcacgatttgaattaaacggttc	120
pksS mutant	1	-----	1
pksS corrected	121	ggagccacatatccggcctggttaattacccgatacgaatgattgtatggcctttttaaaa	180
pksS mutant	1	-----	1
pksS corrected	181	gacaatcgaattacaagagacgtaaaaaatgtgatgaaccaagaacaaatcaaaatgctc	240
pksS mutant	1	-----	1
pksS corrected	241	aacgttagtgaagatatcgattttgtatccgatcatatgctggcaaaagacacacctgac	300
pksS mutant	1	-----	1
pksS corrected	301	catacccgctgagatcacttgttcatcaagcatttactccccgaaccattgaaaatctg	360
pksS mutant	1	-----	1
pksS corrected	361	cgcggcagcattgaacaaattgctgaacagcttttagatgaaatggaaaaagaaaataaa	420
pksS mutant	1	-----	1
pksS corrected	421	gcggatatcatgaaatccttcgcttcccccttgccttttattgttatatctgaattgatg	480
pksS mutant	1	--ggatatcatgaaatccttcgcttcccccttgccttttattgttatatctgaattgatg	58
pksS corrected	481	ggaatcccaaaaagaagatcggtcacagtttcaaactctggaccaatgcgatggttgatacc	540
pksS mutant	59	ggaatcccaaaaagaagatcggtcacagtttcaaactctggaccaatgcgatggttgatacc	118
pksS corrected	541	tctgaaggtaatagagagctgacaaatcaggcccttcgtgaatttaaagattatatcgct	600
pksS mutant	119	tctgaaggtaatagagagctgacaaatcaggcccttcgtgaatttaaagattatatcgct	178
pksS corrected	601	aagctgatccatgacagaagaataaaagccaaaagacgatttaatacagcaaaacttgtgcat	660
pksS mutant	179	aagctgatccatgacagaagaataaaagccaaaagacgatttaatacagcaaaacttgtgcat	238
pksS corrected	661	gctgaggaaaaacggcagcaagttaagcgaa	720
pksS mutant	239	gctgaggaaaaacggcagcaagttaagcgaa-----	268
pksS corrected	721	gttgtagccggccttgaacaactgttaacttactcggctcaggcaccctcgcattgctg	780
pksS mutant	268	-----	268
pksS corrected	781	cagcacaagaaggaatgtgagaagctcaagcagcagcctgaaatgatcgctacagcggtt	840
pksS mutant	268	-----	268
pksS corrected	841	gaagaattgctcgatacacctcacctgtcgttatgatggcaaatcgggtgggccatcgaa	900
pksS mutant	268	-----	268
pksS corrected	901	gactttacatataaggggcattcgatcaaaagaggagacatgatttttataggcatcgga	960
pksS mutant	268	-----	268
pksS corrected	961	tctgccaatcgcgaccgaatttttttgagaaccccgaaatattaataataaatcggtcg	1020
pksS mutant	268	-----	268
pksS corrected	1021	cctaatagacatatttcttttggttttgccattcatttctgcttaggagcgctcttgcc	1080
pksS mutant	268	-----	268
pksS corrected	1081	aggctggaaggccacattgcatttaaagcacttttgaagagatttctgatattgaactt	1140
pksS mutant	268	-----	268
pksS corrected	1141	gcggttgacactgatgacattcaatggagaaaaaatgtctttttaagaggattagaaagt	1200
pksS mutant	268	-----	268
pksS corrected	1201	ctccctgtttcactttcaaaataa	1224
pksS mutant	268	-----	268

After running a PCR with the primers, pksS MUTINUP and pksS MUTINDN, a gel electrophoresis was used to analyze the products, shown in figure III.16. After purification, the mutated gene and pMUTIN4 plasmid were digested, shown in figure III.17, and then ligated together by T4 DNA ligase. Six different concentrations of the insert were used to ensure successful ligation into the vector. Each ligation reaction was then transformed into competent Nova Blue cells and plated onto agar plates.

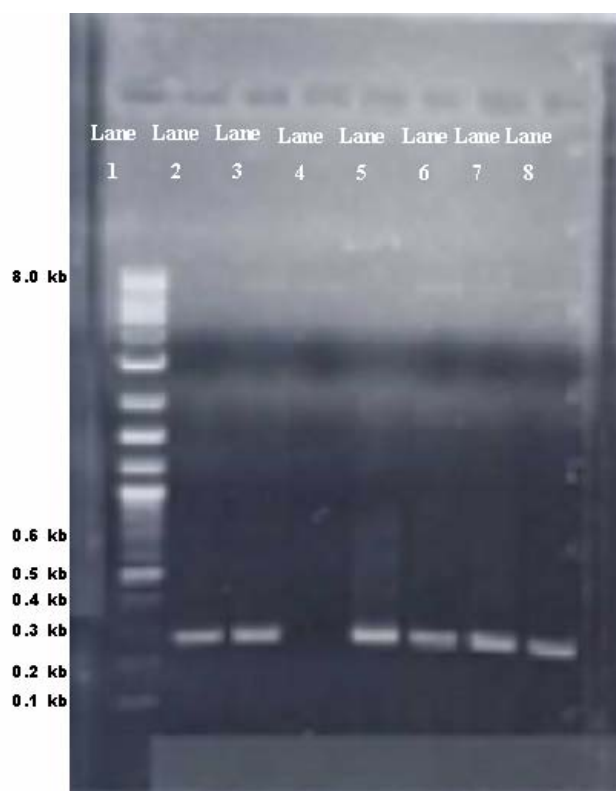
FIGURE III.16. Agarose gel of PCR product using pksS MUTINUP and pksS MUTINDN.



Following an overnight incubation, the colonies were used to inoculate starter cultures. After purifying the starter cultures, the plasmids were screened for the insert. The first set of plasmids were screened using a restriction digest. After digesting the plasmids with *HindIII* and *SacI*, the samples were analyzed using a 0.7% agarose gel.

Because the gel electrophoresis was difficult to analyze, a PCR was run on each plasmid using BS168 as a positive control. The gel electrophoresis, figure III.17, showed positive hits for 2₃, 5₂, and 7₂. The positive hits were then used in the transformation into *Bacillus subtilis* strain OKB105.

FIGURE III.17. PCR of mutated plasmids, Lane 1: Molecular weight, Lane 2: 1₁, Lane 3: 2₃, Lane 4: 3₁, Lane 5: 5₂, Lane 6: 7₂, Lane 7: 10₃, Lane 8: BS168 genomic template

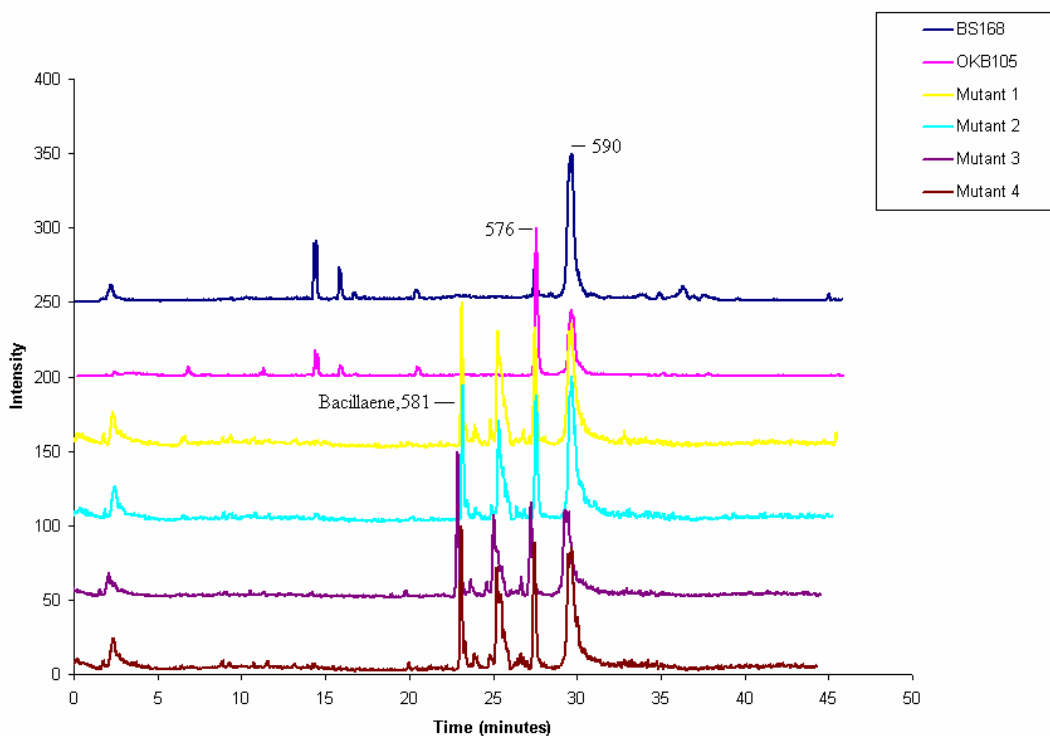


Following the transformation into *Bacillus subtilis*, detailed in III.E.5, the genomic DNA plasmids were screened for insertion into the genome using PCR. Using gel electrophoresis, four positive hits, 2₃, 7₂, 10₃ were found for the successful transformation of the mutated version of *pksS*/pMUTIN4 into *Bacillus subtilis*.

III.C.5. Characterization using *pksS* mutant, methanol, and LCMS

Following the identification of 4 viable transformants containing the mutated version of *pksS*, the mutants were grown in Landy media for 2 days. Each sample was analyzed by methanol extraction and applied to the Hewlett Packard Agilent 1100 series HPLC with a 5 μ M Prevail C-18 column attached. Chromatograms were collected using the LCQ Advantage Thermo Finnigan liquid chromatography coupled with electrospray ionization mass spectrometry detection. The following figure, figure III.18, shows the mutants and controls extracted by methanol after a 48 hour growth period. The two controls, BS168 and OKB105, show two peaks of 590 and 576. The polyketide or compounds that these peaks correlate to are unknown. The next four peaks show a distinct peak at 581, possibly corresponding to bacillaene. This peak is absent in the controls of BS168 and OKB105. It is possible that in presence of PksS, bacillaene is not produced and some other polyketide is being synthesized.

FIGURE III.18. LCMS Chromatograms for Mutants of PksS in MeOH after 48 hour growth period.



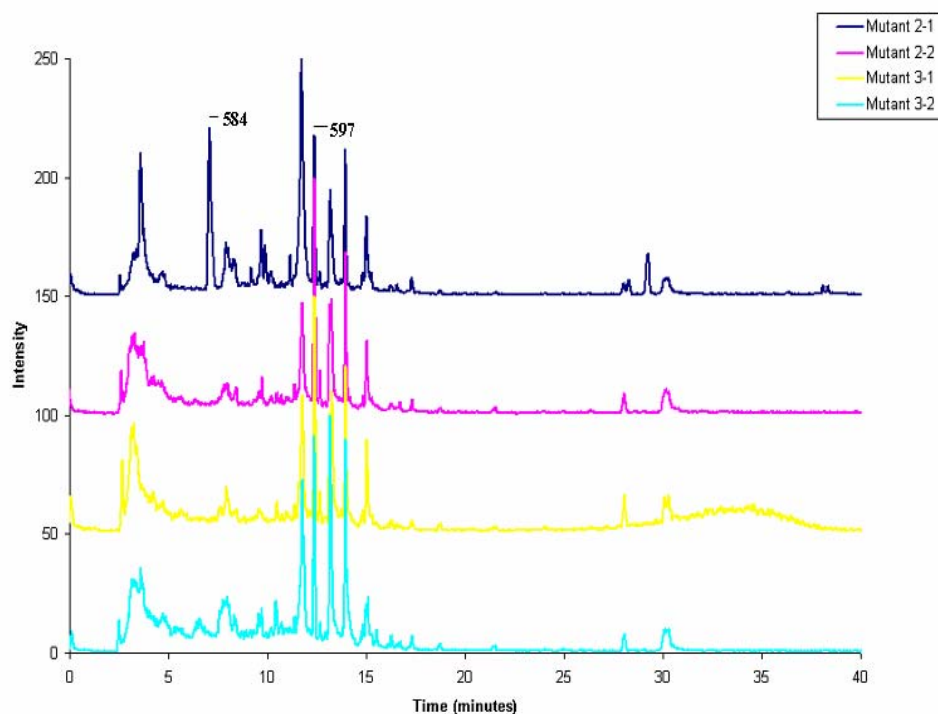
III.C.6. Characterization using *pksS* mutant, acetonitrile, acetic acid, and LCMS

After several runs of the mutated PksS using methanol for extraction, the 5 μ M Prevail C-18 column experienced a clog and was no longer functional. After the arrival of a new column, a new method was chosen in hopes to prevent any precipitation of protein during the analysis.

To prevent the precipitation, methanol was replaced by a solution of 95:5 acetonitrile: 1% acetic acid solution. The mutants 2₁, 2₂, 3₁, and 3₂ were grown for 48 hours and then triturated with the new solvent. The samples were applied to the HPLC-

MS and analyzed as before. Figure III.19 shows the chromatograms obtained after a 45 minute analysis of each sample. The chromatograms show several distinct peaks at 597 and 584. Although these masses do not correspond to a known polyketide, it is possible the can be correlated to some unknown substrate for PksS.

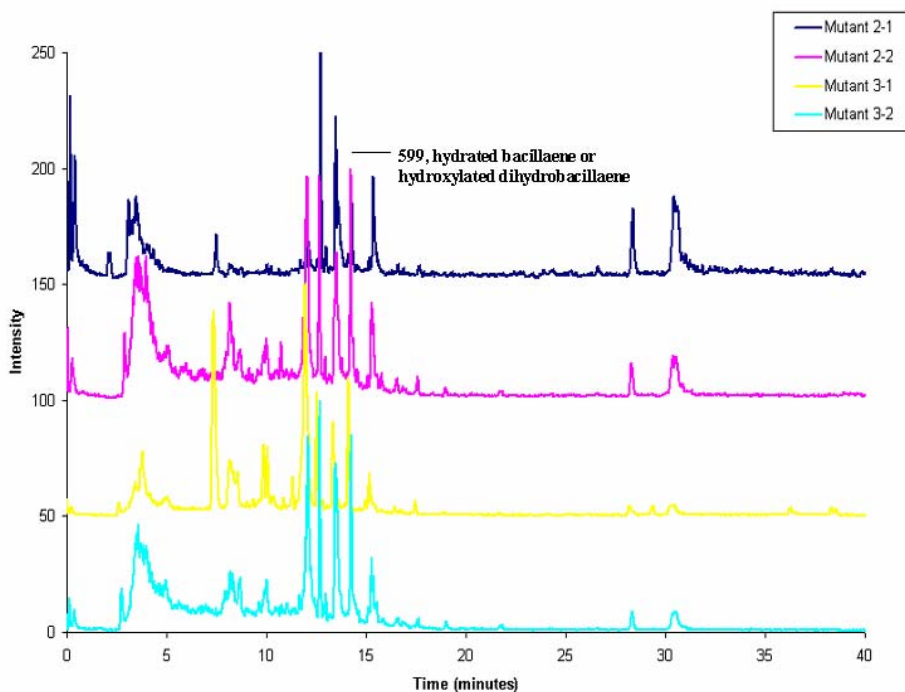
FIGURE III.19. PksS mutants extracted by 95:5, acetonitrile: 1% acetic acid solution after a 48 hours growth period



The cultures were allowed to continue to grow for another 24 hours, to achieve a total growth period of 72 hours. The primary goal was to determine if there was any effect of the growth period of the polyketides that were being produced. Figure III.20

shows that after an extra 24 hours of incubation, a distinct peak at 599 arises. This peak may correspond to the production of hydroxylated bacillaene or hydrated dihydrobacillaene. Assuming that *pksS* has been knocked out through mutations, it is not understood why a peak at 599 m/z is present.

FIGURE III.20. PksS mutants extracted by 95:5 acetonitrile: 1% acetic acid solution after a 72 hours growth period.



In another attempt to determine any possible effects on the extraction of a polyketide, OKB105 was grown in different media. Although OKB105 is a control, it is important to know the effect of the growing media on the desired polyketide. Figure III.21 and figure III.22 shows that there is a significant difference in the masses produced

from the different types of media. The issue, however, is that the masses obtained do not correspond to any expected polyketide.

FIGURE III.21. OKB105 grown for 48 hours in Landy Media.

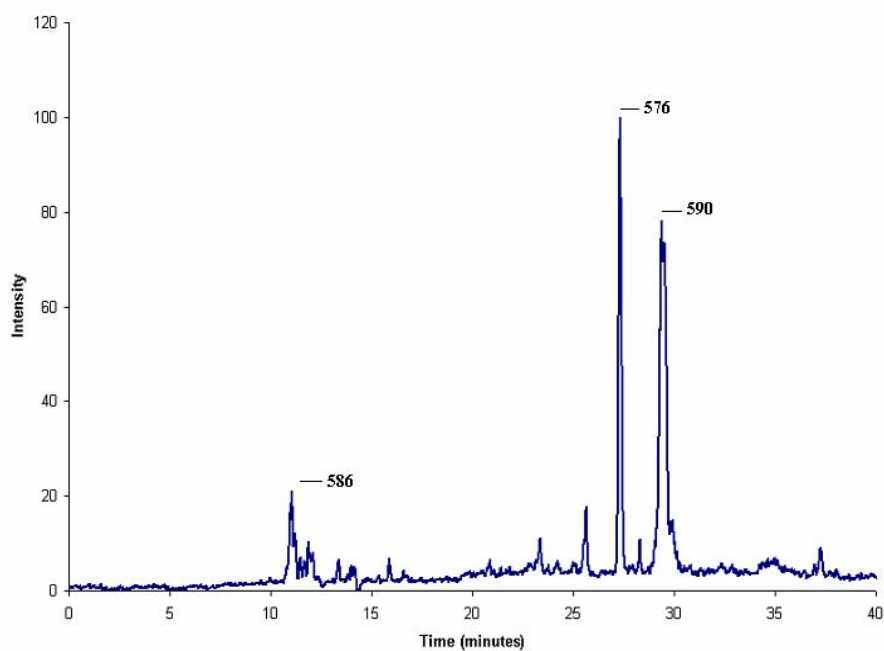
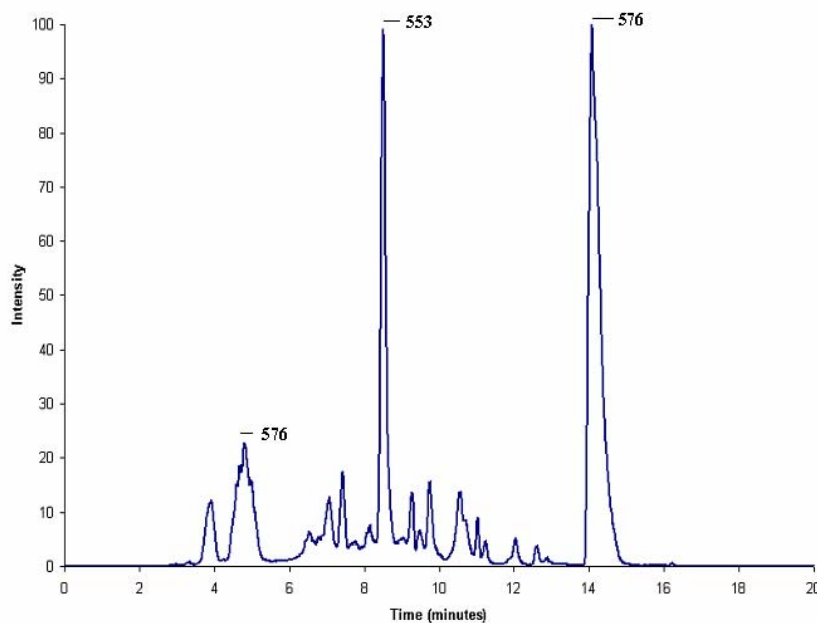


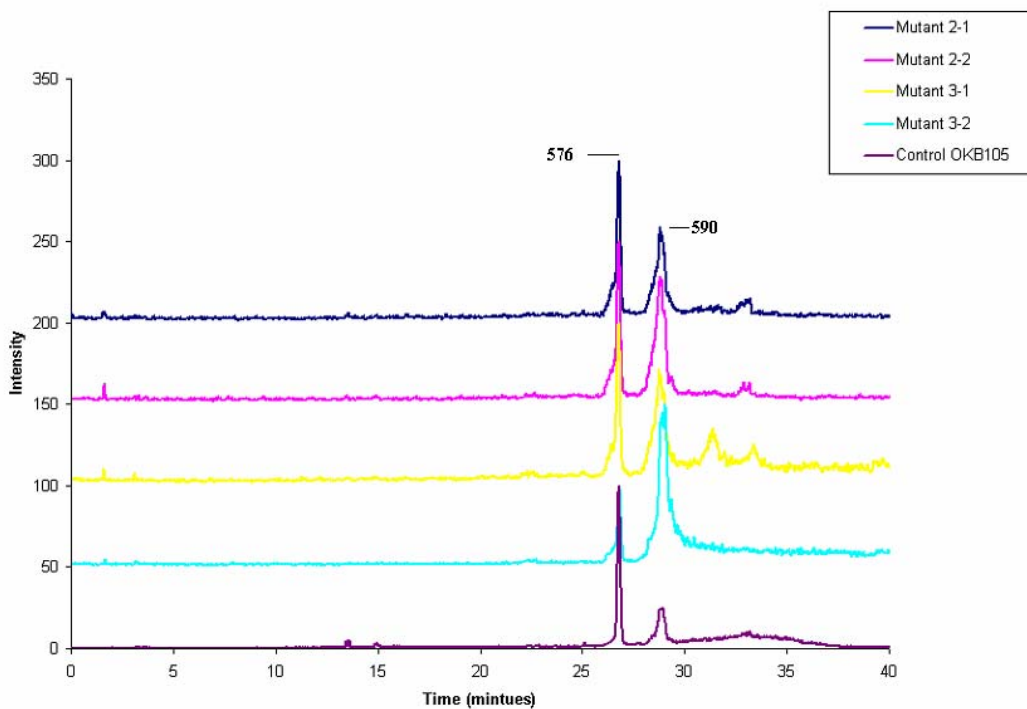
FIGURE III.22. OKB105 grown for 48 hours in LB media



III.C.7 Characterization using *pksS* mutant, ethyl acetate, and LCMS

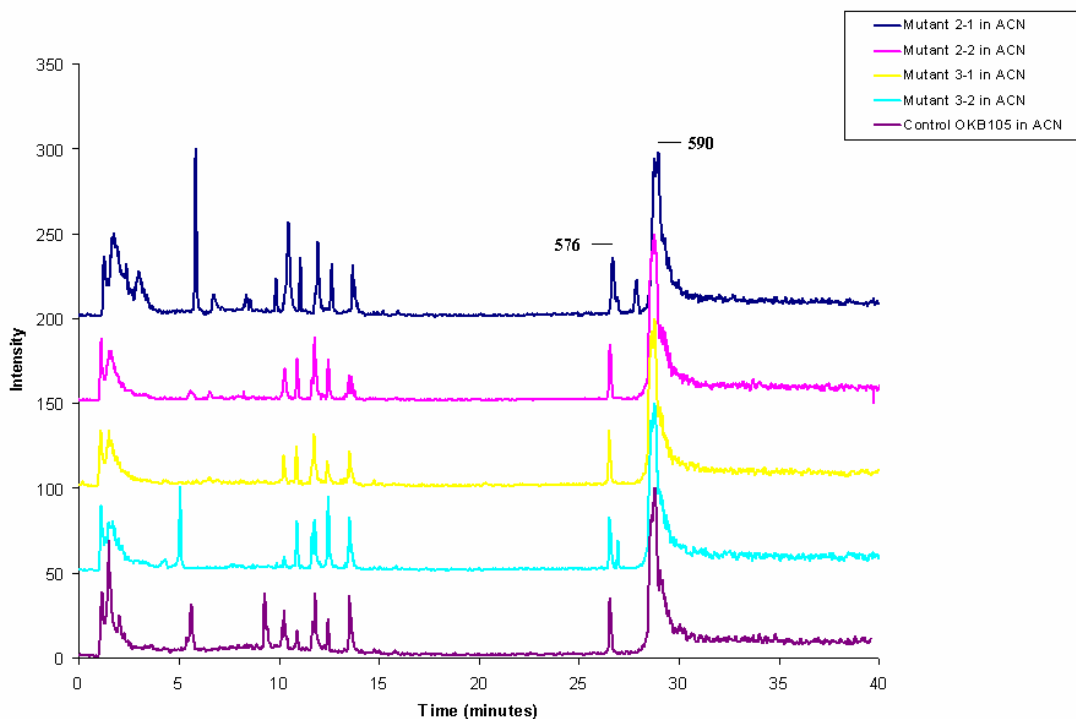
After receiving results that may correlate to the failed extraction of hydrated dihydrobacillaene or hydroxylated bacillaene, a new extraction solvent was used. After triturating the lyophilized extracts with ethyl acetate, it was determined that the extract was insoluble in ethyl acetate. The supernatant obtained from this extraction was applied to the HPLC-MS and analyzed for any production of a substrate. Figure III.23 shows two distinct peaks of 576 and 590, possibly corresponding to the extraction of plastic from the microcentrifuge tubes used. These masses show that no polyketides were extracted that corresponded to substrates of our interest.

FIGURE III.23. PksS mutants extracted in ethyl acetate after a 48 hour growth period



After failing to receive any results for the extraction of substrates by ethyl acetate, the pellets were then resuspended in 95% acetonitrile:5% 1% acetic acid. The supernatant obtained from the resuspension was applied to the HPLC-MS and analyzed for the production of any compounds. Figure III.24 shows that no masses were obtained that correlated to the desired polyketides, therefore, extraction by ethyl acetate was not successful.

FIGURE III.24. PksS mutants after resuspension in ethyl acetate and acetic acid: acetonitrile after a 48-hour growth period



III.D. Conclusion

After determining a critical error in the published sequence of *pksS*, it was important to determine if *pksS* would act as a cytochrome P450. With the use of the carbon-monoxide complex assay, it was possible to identify PksS as a cytochrome P450.

With the combination of PksS with a reductase, sodium dithionite, and carbon monoxide, it was determined that PksS was indeed a cytochrome P450. The characterization was achieved by monitoring the shift in wavelengths from 420 nm to 450 nm. A shift could be seen using purified PksS and crude PksS resuspended in 50 mM potassium phosphate, pH 8.0.

After the successful determination that PksS is a cytochrome P450, the next step was to determine a suitable reductase for the reduction of the heme center. Testing different strains, BS168 and OKB105, and different electron donors, NADH and NADPH, led to the establishment of a suitable reductase system. The addition of NADH and BS168 to PksS under anaerobic conditions resulted in the reduction of the heme center and a change in wavelength. The shift from 420 nm to 450 nm allowed for the identification of BS168 protein extract as reductase for PksS. However, the addition of BS168 extract and NADPH did not result in the reduction of the iron center. This information leads to the assumption that PksS is a member of Class I, NADH-dependent cytochrome P450s (Ortiz de Montellano 2005). Also, there was no reduction of the heme upon the addition of OKB105 and either NADH or NADPH.

To determine the role of PksS in the biosynthesis of bacillaene or a precursor, the polyketides needed to be extracted from a known strain, BS39374 that carries the desired polyketide. Using methanol extraction, two controls were used to compare to the production of a substrate upon the addition of PksS, an electron donator, and a reductase. The chromatograms show that in all samples, controls and full reactions, peaks correlating to hydrated bacillaene or hydroxylated bacillaene, bacillaene, and dihydrobacillaene are recorded. The samples undergoing a full reaction show the disappearance of the peak correlated to dihydrobacillaene. This leads to the preliminary assumption that the substrate for PksS is dihydrobacillaene.

To obtain more information concerning the function of PksS in the biosynthesis of bacillaene, a mutant of *pksS* was constructed. After efficiently cloning a position of *pksS*,

into the pMUTIN4 vector, then *pksS*/pMUTIN4 was transformed into competent Nova Blue (*E. coli*) cells. The plasmids were screened for the insert by a restriction digest and PCR. The plasmids 2₃, 5₂, and 7₂ were found to contain the insert and were transformed into *Bacillus subtilis*. Following the transformation, the genomic DNA from three mutated strains were screened again using PCR. The positive hits, 2₃, 7₂, and 10₃, were found to contain the mutated inserts.

After obtaining extracts that contained the mutated *pksS*, the extracts from mutants and wild type strains were applied to the HPLC-MS to determine the role of PksS. The first method, which used methanol as the extraction solvent, resulted in peaks that correlated to desired polyketides. The controls, BS168 and OKB105, showed peaks of 590 and 576, which correspond to unknown compounds. The mutants showed distinct peaks at 581, which corresponded to bacillaene. This peak is absent in the controls of BS168 and OKB105, possibly showing that bacillaene is not produced to a significant amount when PksS is functional.

To further the understanding the role of PksS and prevent the possibility of protein precipitation on the column, a different solvent was used. A 95% acetonitrile:5% 1% acetic acid solution was used to triturate the mutants after a 48-hour incubation. The chromatograms showed several distinct peaks at 597 and 584. Although these masses did not correspond to a specific polyketide, it is possible they can be correlated to some unknown substrate for PksS. After increasing the incubation period to 72 hours, distinct peaks arose at 599 corresponding to the production of hydroxylated bacillaene or hydrated dihydrobacillaene. This peak should not have been present in any mutated

versions of *pksS* if PksS hydroxylates the substrate. We assume that the mutation may not have worked.

Another solvent, ethyl acetate, was also used in an attempt to successfully extract the polyketide. Although the pellets were not soluble in ethyl acetate, the samples were still applied to the HPLC-MS. Chromatograms showed two distinct peaks of 576 and 590, providing insight that no polyketides were extracted that corresponded to substrates of our interest. The insoluble material was then resuspended in 95% acetonitrile:5% 1% acetic acid and applied once again to the HPLC-MS. Again no information was obtained that would provide insight on the role of PksS in the biosynthesis of bacillaene or dihydrobacillaene.

III.E. Future work

The results received during the analysis of the mutated *pksS* showed no consistent results. Although several runs were attempted and analyzed, it was difficult to establish controls for the mutations. A full reaction containing mutated PksS, an electron donor, a reductase, and an extract of bacillaene was attempted. During this process, we experienced protein precipitation during the analysis with LCMS. Our time was then dedicated to determining an efficient way to extract the mutants without causing protein precipitation.

To complete the characterization of PksS, future work will be dedicated to determining appropriate extraction methods. A solid phase extraction method has been reported by Butcher et. al. (2007), in which the extract was successfully obtained and

analyzed using NMR techniques. Future work will also be focused on a more stringent method of determining the correct incorporation of the mutation into the genome.

III.F. Experimental

III.F.1. P450 Assay

The cytochrome P450 assay was performed using two different suspensions of PksS. In the first method, PksS was extracted for the cell culture after induction with IPTG (Sigma). Every hour, following induction, 10 mL of media were extracted from the culture and resuspended in 3 mL of 50 mM potassium phosphate, pH 8.0. The cells were then divided into one-thirds and placed into cuvetts. Controls were also set up to determine the effect of carbon monoxide, potassium phosphate, pH 8.0, and the sodium dithionite on PksS. The first control was a 1 mL mixture containing crude cells from the culture resuspended in 50 mM potassium phosphate, pH 8.0 and run through the Varian CARY 100 Bio UV-Visible Spectrophotometer. The spectrophotometer scanned between 400 nm to 500 nm for P450 activity (Phillips 1998). After determining that there was no P450 activity, shown by a strong peak at 420 nm, the next control was set up. In the second control, one milliliter of the suspension was purged with carbon monoxide for 1 minute. The cuvet was then placed into the UV/VIS and scanned for any P450 activity (Phillips 1998). After determining the carbon monoxide had no effect when combined separately with PksS, the final control was set up. In this control, 1 mL of crude PksS resuspended in 50 mM potassium phosphate buffer, pH 8.0, was combined with 5 mg of sodium dithionite (Phillips 1998). After scanning the mixture for any P450

activity, it was determined that the components had no effect on PksS when combined separately.

After performing several controls, 1 mL of the PksS and 50 mM potassium phosphate, pH 8.0, was purged for one minute. Following the purging, 5 mg of sodium dithionite was added to the cuvet and gently inverted to mix. The sample was then placed into the cuvet and scanned for any P450 activity (Phillips 1998). After obtaining a spectra showing a strong wavelength shift from 420 nm to 450 nm, it was determined that PksS was a cytochrome P450.

The next step in the cytochrome P450 assay was to use PksS purified by a Ni-NTA column (Qiagen). Before running the reactions, the protein was run through a Sepharose G25 column (Sigma-Alrich), molecular weight range of 100-5,000, to remove any residual imidazole from the purification process. Controls were set as before, monitoring the effects of sodium dithionite, carbon monoxide, and the 50 mM potassium phosphate, pH 8.0.

The first control monitored the effects of potassium phosphate on PksS. To monitor this effect, 150 μ L of PksS was combined with 850 μ L of 50 mM potassium phosphate, pH 8.0 (Phillips 1998). After scanning the mixture for any P450 activity, it was determined that 50 mM potassium phosphate had no visible effects on the purified PksS. The second control monitored the effects of the addition of sodium dithionite to PksS resuspended in 50 mM potassium phosphate. The sample was prepared by combining 150 μ L of PksS, 850 μ L of 50 mM potassium phosphate, pH 8.0, and 5 mg of sodium dithionite (Phillips 1998). The sample was then placed into the UV/VIS

Spectrophotometer and scanned from 400 nm to 500 nm for any possible P450 activity. After obtaining spectra showing no shift in wavelength from 420 nm to 450 nm, it was determined that the addition of sodium dithionite had no effect on PksS. The final control was used to determine if the addition to carbon monoxide to resuspended PksS would cause a shift in the wavelength. The control was performed by combining 150 μ L of PksS and 850 μ L of 50 mM potassium phosphate, pH 8.0, in a cuvet and then purging the system for approximately one minute. After purging, the cuvet was placed into the spectrophotometer and scanned for any reduction of PksS (Phillips 1998). After determining the carbon monoxide had no effect on PksS, the full reaction was initiated.

To perform a cytochrome P450 assay on PksS, 150 μ L of PksS, 850 μ L of 50 mM potassium phosphate were combined and purged with carbon monoxide for one minute. After purging with carbon monoxide, 5 mg of sodium dithionite were added and the cuvet was gently inverted to mix the sample. The cuvet was then placed into the spectrophotometer and scanned for P450 activity (Phillips 1998). The spectra showed a strong peak at 450 nm, representing the reduction and binding of carbon monoxide to the iron center.

III.F.2 Assay for PksS reductase activity

After classifying PksS as a cytochrome P450, several strains derived *Bacillus subtilis* were screened for their reduction capabilities. Two specific strains, BS168 and OKB105, were screened because of their genetic capability to produce bacillaene through polyketide synthesis. To prepare the strains for the reductase activity, starter cultures containing 5 ml of LB were inoculated with a colony of OKB105 and BS168,

respectively. Following inoculation, the cultures were grown in aerobic conditions at 37°C, while being shaken at 240 rpm overnight in the New Brunswick Scientific C24 Incubator Shaker. The starter cultures were then used to inoculate different type of growing media to determine the optimal media. One of the media used was a minimal media containing 1x Landy Salts, 2.0% D-glucose (Difco Laboratories), and 0.5% l-glutamic acid (Fisher Science). The other media was LB, containing 1.0% Bacto-tryptone (Difco Laboratories), 0.5% Bacto-Yeast (Difco Laboratories), and 1.0% NaCl (Difco Laboratories). Starter cultures were used to inoculate each of the medias and the cultures were allowed to grow for 2 days at 37°C at 240 rpm. After growing both strains in the media, it was determined that a nutrient rich media was efficient in the production of the reductase. Any experiments carried out for the reductase utilize LB as the growing media.

Following the two-day incubation period, both strains were centrifuged at 8,500 rpm at 4°C for 30 minutes. The pellet was then resuspended in 50 mM potassium phosphate, pH 8.0, and lysed on ice using lysozymes (Fisher Science) supplied by Dr. Gregory Raner. The suspension was then lysed further by sonication, using the Fisher Scientific 60 Sonic Dismembrator, and centrifuged at 11,500 rpm at 4°C for 30 minutes. The supernatant was then removed and filtered through a 0.45 µm membrane (Fisher Science). PksS was extracted using the methods described in Chapter II.E.8.

Samples were prepared for the reaction by combining 8 µM of PksS and 330 µM of NADH/NADPH (Sigma/Alexis Biochemicals). The samples were brought to a final volume of 3 ml using 50 mM potassium phosphate, pH 8.0. After preparing the sample,

the mixture was transferred into a cuvet and sealed with a rubber septum. Using a Schlenck apparatus and needle, the sample was introduced into a controlled, anaerobic environment. To remove any residue oxygen remaining in the cuvet, the system was purged with argon and evacuated using a high-pressure vacuum. After repeating this process three more times, the anaerobic system was purged with carbon monoxide for 15 minutes using a needle and appropriate venting. After purging the sample, the system was scanned for any reduction of PksS. A visible shift in wavelength from 420 nm to 450 nm would ultimately lead to the determination of a suitable reductase for PksS.

To ensure the apparatus was under anaerobic conditions, BM3, a known cytochrome P450 capable of complete reduction with the addition of carbon monoxide, was used as a control. BM3, supplied by Dr. Gregory Raner, contains a reductase domain along with the P450 domain and is therefore capable of accepting electrons directly from a cellular reductant. In order to set up the control, 8 μ M of BM3 was combined with 83 μ M of NADPH. The system was then sealed with a rubber system and purged with argon. After purging the sample, the system was then evacuated using a high-pressure vacuum. After repeating this process three times, the sample was then purged with carbon monoxide for 15 minutes via a needle and a ventilation needle. After scanning the sample for the reduction of the heme, it was determined that the apparatus was set up correctly.

III.F.3. Determining function of PksS using LCMS

To determine the function of PksS in the polyketide biosynthesis of bacillaene, crude extracts must be obtained from BS39374, a strain derived from *Bacillus subtilis*. To prepare the strain for extraction of bacillaene, a starter culture of BS39374 was in LB overnight at 37°C at 240 rpm. The starter culture was used to inoculate a 50 mL culture of Landy media. The culture was incubated at 37°C for 72 hours at 240 rpm. Bacillaene was extracted by combining 0.500 ml of the culture and 1 ml of HPLC-grade methanol (Pharmaco). The sample was then vortexed for 20 seconds using the Vortex Genie and then centrifuged at top speed for 5 minutes in the Eppendorf Centrifuge 5415 C. The supernatant was extracted and the methanol was removed by centrifugation extraction using the Savant Speed Vac SC110. The system was evacuated using a Savant ValuPump VLP 120 while being maintained at a low temperature by the Refrigerated Vapor Trap RVT400 (by Savant).

Reductase strains, OKB105 and BS168, were prepared by inoculating starter cultures containing LB. After an overnight incubation at 37°C at 240 rpm, the cultures were used to inoculate 500 mL LB cultures. These cultures were grown at 37°C for 48 hours at 240 rpm. The reductases were extracted by centrifugation at 8,500 rpm for 30 minutes at 4°C in the Beckman Avanti J-25 Centrifuge. The pellet was resuspended in 10 ml of 50 mM potassium phosphate buffer, pH 8.0, and lysed on ice using lysozymes. The suspension was also lysed by sonication. After breaking down the cells walls, the media was centrifuged at 11,500 rpm for 30 minutes at 4°C. The supernatant was used for the subsequent reactions.

Samples used on the Liquid Chromatography Mass Spectrometer were prepared by combining 25.0% PksS, 10.7% of either NADH/NADPH, 10.7% of reductase, BS168 or OKB105, and 53.6% of the bacillaene methanol extract to a total volume of 600 μ L. After combining the respective components, each sample was incubated at room temperature for 1 hour and then filtered using a 0.45 μ m membrane. The samples were then transferred into Target DP™ Vials C4000-1 (National Scientific Company) and sealed with 9 MM Blue Caps T/S (Fisherbrand). The vials were then placed into the Hewlett Packard Agilent 1100 series HPLC in which the 5 μ M Prevail C-18 column (Alltech) was used. Data was collected using the LCQ Advantage Thermo Finnigan liquid chromatography coupled with electrospray ionization mass spectrometry detector under the following gradient elution: begin at 95:5 1% acetic acid (Fisher Science):acetonitrile (Fisher Science), with the acetonitrile increasing to 95% over a 45 minute linear gradient at 0.2 ml/minute flow rate.

To include each possible control and reaction combination, 13 different reactions were set up. The following table displays the 13 different combinations used to obtain the most accurate data.

TABLE III.1 Reactions used in LCMS

	Bacillaene MeOH, NSV	Bacillaene MeOH	PksS	BS168	NADH	NADPH
Control 1	-	+	-	-	-	-
Control 2	-	+	-	+	+	-
Control 3	-	+	+	-	+	-
Control 4	+	-	-	-	-	-
Full rxn	-	+	+	+	+	-
Full rxn	-	+	+	+	-	+

MeOH : methanol

NSV : no speed vac

III.F.4. Construction of *pksS* mutants

After the obtaining inconclusive results for the methanol extraction of bacillaene, mutants of *pksS* were designed in hopes of determining the role of PksS in the biosynthesis of bacillaene. Upstream and downstream primers were designed to amplify a 268 bp section of *pksS*. The upstream primer pksSpMUTINUP and downstream primer pksSpMUTINDN were combined with Taq polymerase, dNTPs, and BS168 as the genomic template. The PCR was run at the following conditions: 94.0°C for two minutes; 30 cycles of 30 seconds at 94.0°C, 30 seconds at 60.0°C, and 90 seconds at 72.0°C; then a hold at 4.0°C. The PCR product was then analyzed by gel electrophoresis. After determining the product was the correct size, the product was purified using the “QIAquick PCR Purification Kit.”

The PCR product and pMUTIN4 plasmid were then digested separately with the restriction enzymes *HindIII* and *SacI* for 3.5 hours at 37.0°C. To inactivate the enzymes, the samples were then placed in a 65.0°C water bath for 20 minutes (Vagner 1998).

After incubation, a 0.7% low melting agarose gel was used to purify and measure the size of the material. The bands were excised from the gel and incubated in a water bath at 75°C. The products were then combined at three different concentrations described here: The insert:plasmid ratio for the 1st reaction was 2µL:1µL, for the second reaction 5µL:1µL, and for the 3rd reaction 1µL:2µL. Each reaction was mixed with water, Ligase buffer, and T4 DNA ligase. The samples were placed in a thermocycler overnight with the following conditions: 30 minutes at 37.0°C, 2 hours at 23.0°C, and then 4.0°C for the remainder of the incubation. The following day, each mixture was transformed into competent DH5α (*E. coli*) cells by combining 20µL of the ligation mixture to 100µL of the chemically competent cells. The cells were incubated on ice for 30 minutes followed by a 2 minute incubation at 37°C. The samples were then incubated at room temperature for 10 minutes and then resuspended in 1 mL of LB media and incubated at 37.0°C for 1 hour. Following the incubation, the cells were plated on agar plates containing 25 µg/ml of ampicillin and 30 µg/mL of kanamycin and incubated overnight at 37.0°C.

The cloning plasmid, pMUTIN4, was prepared by inoculating two starter cultures containing 5 mL of LB media and 25 µg/mL of ampicillin with pMUTIN4/DH5α. The starter cultures were grown at 220 rpm overnight at 37°C. The following day, the cultures were purified using the “QIAGEN MiniPrep Kit.” The DH5α cells were made competent by inoculating a starter culture containing LB media with a colony of DH5α

and then grown at 220 rpm overnight at 37°C. The cultures were then spun down in a clinical centrifuge at maximum speed and then resuspended in 3 mL of CaCl₂. After incubating the cells on ice for 30 minutes, the culture was centrifuged again and then resuspended in 1 mL of CaCl₂.

After several failed attempts to grow the pMUTIN4/mutated *pksS* in DH5 α , it was determined that the host cell had expired. The next attempt utilized a different host cell, Nova Blue, for the overexpression of the mutated *pksS*.

Nova Blue, which does not cause any sequence changes or contain any antibiotic resistance, was streaked onto an agar plate containing LB media. After an overnight incubation at 37.0°C, a starter culture containing LB media was inoculated using a colony. The culture was then grown overnight at 220 rpm at 37.0°C. The cells were made chemically competent the following day using CaCl₂.

To prepare for transformation of pMUTIN4/mutated *pksS* into Nova Blue, the mutated *pksS* and pMUTIN4 were digested using *HindIII* and *SacI*. The digested material were then analyzed and purified using a 0.7% low melting agarose gel electrophoresis. The DNA was excised and heated at 75°C to melt the gel. The products were then combined at three different concentrations: The insert:plasmid ratio for the 1st reaction was 2 μ L:1 μ L, for the second reaction 5 μ L:1 μ L, and for the 3rd reaction 1 μ L:2 μ L. Each reaction was mixed with water, Ligase buffer, and T4 DNA ligase. The samples were placed in a thermocycler overnight with the following conditions: 30 minutes at 37.0°C, 2 hours at 23.0°C, and then 4.0°C for the remainder of the incubation. The following day,

each mixture was transformed into competent Nova Blue cells by combining 20µL of the ligation mixture to 100µL of the chemically competent cells.

The plasmids were transformed into the host cell by a series of heat and cold shock incubations. The samples were then combined with 1 mL of LB and incubated at 37°C for 1 hour. The cells were then centrifuged at 8,000 rpm for 5 minutes, resuspended, and then plated on agarose plates containing 25 µg/mL of ampicillin. The plates were incubated at 37°C overnight. Several starter cultures were inoculated using colonies from each concentration of the ligation mixtures and then purified using the “QIAprep Spin Miniprep Kit.”

The products were screened for the mutated insert acceptance using a restriction digest and PCR. Although the restriction digest was not conclusive, the PCR screening was also used. Positive hits were recorded for mutated *pksS* and pMUTIN4 containing the following concentrations of insert:plasmid: (3) 2 µL:1 µL, (5) 5 µL:1µL, (6) 5µL:2µL, (7) 10µL:1µL. The purified plasmids were then retransformed into competent Nova Blue cells and plated on agar containing 25 µg/mL of ampicillin. Starter cultures containing 5 mL of LB media and 25 µg/mL of ampicillin were inoculated with the colonies from the previous reaction and grown overnight at 37°C at 220 rpm. Following the incubation, the cultures were purified using the “QIAGEN Miniprep Kit.”

III.F.5 Transformation of mutant *pksS* into *Bacillus subtilis*

After obtaining plasmids with positive hits for the mutated insert, the plasmids were then transformed into competent *Bacillus subtilis* strain 168 cells. To prepare the *Bacillus subtilis* 168 cells for transformation, an agar plate containing LB media was

streaked with a stock solution of BS168 cells. The plate was placed in the incubator at 37°C for an overnight incubation. Following the incubation, a starter culture containing 5 mL of LB media was inoculated using one colony from the plate. The culture was then placed in aerobic conditions at 37°C overnight at 220 rpm. The culture was centrifuges at top speed for 5 minutes and then resuspended in 1 mL of minimal media #1 (Anagnostopoulos 1961).

The composition of minimal media #1 was: 0.2% ammonium sulfate, 1.4% dipotassium phosphate, 0.6% monopotassium phosphate, 0.1% sodium citrate•2H₂O, 0.02% magnesium sulfate•7H₂O, containing 0.5% glucose, 50 µg/mL L-Tryptophan, and 0.02% casein hydrolyzate acid (Anagnostopoulos 1961).

The solution was centrifuged again in a clinical centrifuge at top speed for 5 minutes and then resuspended in 1 mL of fresh minimal media #1. The cells were then incubated in a tube with a diameter of 17 mm at a 45° angle for 4 hours at 37°C. The cells were then centrifuged for 5 minutes at 3,000 rev/minute and resuspended in a 10-fold dilution of fresh minimal media #2 (Anagnostopoulos 1961).

Minimal media #2 is comprised of 0.5% glucose, 50 µg/mL L-Tryptophan, 0.01% casein hydrolyzate acid, 0.2% ammonium sulfate, 1.4% dipotassium phosphate, 0.6% monopotassium phosphate, 0.1% sodium citrate•2H₂O, 0.02% magnesium sulfate•7H₂O, and an additional 5 µmoles/mL of magnesium sulfate•7H₂O (Anagnostopoulos 1961).

The 10 mL samples were divided into 1 mL samples and transferred into fresh tubes with the appropriate diameter. The plasmids purified from the Nova Blue cells were then added separately to the tubes. The following insert:plasmid concentrations

obtained in the previous reaction will be renumbered as follows: (1₁) to 1, (2₃) to 2, (3₁) to 3, (5₂) to 5, (7₂) to 7, (10₃) to 10. In order to achieve approved transformation, 1 µL and 2 µL of the purified plasmid were added to the competent BS168 cells. To identify these samples, a subscript of either one or two denoting 1 µL or 2 µL will be added to the previous numbering. The new numbering of the samples is as follows: 1₁, 1₂, 2₁, 2₂, 3₁, 3₂, 5₁, 5₂, 7₁, 7₂, 10₁, 10₂. The samples were then incubated at a 45° slant for 90 minutes at 37°C. Following the incubation, the samples were centrifuges for 5 minutes at top speed and 950 µL of the supernatant was removed. The transformants were resuspended in the remaining supernatant and then plated by using 20 µL of sample onto agar plates containing erythromycin and LB media. The plates were incubated overnight at 37°C. Colonies obtained from transformation were used to inoculate separate starter cultures containing 5 mL of MB media and 0.3 µg/mL (Anagnostopoulos 1961).

After the overnight incubation, 8 starter cultures were chosen at random to be purified using the Promega “Wizard Genomic DNA Purification Kit.” The samples purified are as follows: 2₂, 3₁, 5₁, 5₂, 7₁, 7₂, 10₁. To purify the samples, 1 mL from each starter culture was centrifuged separately in a clinical centrifuge at top speed for 5 minutes. The pellet was resuspended in 480 µL of 50 mM EDTA and then combined with 120 µL of 5 mg/mL lysozyme solution. The samples were incubated at 37°C for 30 minutes and then centrifuged at top speed for 5 minutes. The pellets were resuspended in 600 µL of Nuclei Lysis Solution and 3 µL of RNase solution. After gently inverting the samples five times, 200 µL of protein precipitation solution was added. The samples were vortexed vigorously for 20 seconds and then incubated on ice for five minutes.

Following the incubation, the samples were centrifuged at 13,200 rpm for 5 minutes and decanted into sterile 1.5 mL centrifuge tubes. To separate the DNA from the protein, 600 μ L of isopropyl alcohol was added to the samples and then centrifuged for 2 minutes. The supernatant, containing the DNA, was carefully decanted and transferred into a new microcentrifuge tube. The tube was gently inverted several times to allow for the precipitation of the DNA and then centrifuged. The supernatant was decanted and the pellet was resuspended in 70% ethyl alcohol. To wash the DNA, the tube was inverted several times and then centrifuged. The supernatant was removed and the DNA was incubated at room temperature to remove any residual ethanol. The DNA was rehydrated by incubating the samples at 4°C overnight after adding 100 μ L of DNA Rehydrating Solution.

Following the 24 hour incubation period, the DNA was screened for proper insertion of the mutated gene using PCR. To screen the plasmid for insert, the plasmids were used as the genomic DNA template, replacing BS168, and combined with the upstream primer, *pksS* UPMUTIN, the downstream primer, *pksS* DNMUTIN, dNTPs, and Phusion polymerase. The PCR products were then screened for mutated *pksS* by gel electrophoresis.

After receiving positive hits for the 2₁, 2₂, 3₁, and 3₂ mutated versions of *pksS*, the transformants were restreaked onto a new plate containing LB media and 0.3 μ g/mL erythromycin. Following an overnight incubation at 37°C, a colony from each plate was used to inoculate starter cultures containing 0.3 μ g/ml of erythromycin. These starter cultures were used to make a stock concentration of the cells. The stock solutions were

made by combining 850 μ L of the culture and 150 μ L of 80% glycerol in a sterile microcentrifuge tube. The stock cells were stored in a -80°C freezer. All positive hits will be used in characterization reactions to determine the role of PksS in the polyketide biosynthesis of bacillaene.

III.F.6. Characterization using *pksS* mutant, methanol, and LCMS

After identifying positive hits of plasmids containing the mutated version of *pksS*, four samples, 2₁, 2₂, 3₁, and 3₂, of the pMUTIN4/mutated *pksS* in BS168 were used to inoculate separate starter cultures containing LB media and 0.3 $\mu\text{g/mL}$ of erythromycin. For a control, two starter cultures contain LB media were inoculated with a colony of BS168 and OKB105. The mutant cultures were grown under aerobic conditions at 37°C for 72 hours at 220 rpm. Following the incubation, 150 μ L of each starter culture was used to inoculate 50 mL overexpression cultures of Landy minimal media. The controls were grown under aerobic conditions at 37°C for 48 hours at 220 rpm. The media contained 1 mL of 1x Landy salts, 1 gram of dextrose, and 0.25 grams of L-glutamic acid. 5 M sodium hydroxide was added to the media to obtain a pH of 6.5.

The samples were analyzed for the effect of the mutated *pksS* by methanol extraction. To extract the polyketide from each sample, 0.5 mL of the culture was removed and transferred into separate 1.5 mL microcentrifuge tubes. The samples were combined with 1.0 mL of HPLC-grade methanol and then vortexed vigorously for 1 minute. The samples were then centrifuged at top speed for 5 minutes and then decanted into clean, separate microcentrifuge tubes. The samples were speed vacuumed at low

temperature to remove any methanol and then syringe filtered using a 0.45 μ m nylon filter. The extracts were transferred into HPLC vials and then analyzed using the Hewlett Packard Agilent 1100 series HPLC in which the 5 μ M Prevail C-18 column (Alltech). Chromatograms were collected using the LCQ Advantage Thermo Finnigan liquid chromatography coupled with electrospray ionization mass spectrometry detection under the following gradient elution: begin at 95:5 1% acetic acid:acetonitrile, with the acetonitrile increasing to 95% over a 45 minute linear gradient at 0.2 mL/minute flow rate.

To test the effect of different growing medias, two starter cultures containing LB media were inoculated with a colony of OKB105. After an overnight incubation at 37°C at 220 rpm, 150 μ L of one starter culture was used to inoculate a 50 mL overexpression culture of Landy Minimal Media. The second starter culture was used to inoculate a 500 mL overexpression culture of LB media. After a two day incubation at 37°C at 220 rpm, samples were removed from both overexpression medias. Bacillaene was extracted using the methanol extraction method and analyzed by HPLC-MS.

III.F.7 Characterization using *pksS* mutant, acetonitrile, acetic acid and LCMS

After several extractions and runs of the methanol extracts on the 5 μ M Prevail C-18 column, the column experienced a fatal clog. Following the order of a new column, new extraction methods were attempted to achieve conclusive results in determining the role of PksS in the polyketide biosynthesis of bacillaene or a precursor.

In an attempt to prevent the precipitation of the extract onto the column, the cultures were combined with the same percentage of solvents, 5% of 1% acetic acid and

95% acetonitrile, used during the analysis of the extracts. To prepare the samples for the new method of analysis, starter cultures containing 5 mL of LB media and 0.3 µg/mL of erythromycin were inoculated with the mutants 2₁, 2₂, 3₁, and 3₂ and grown at 37°C for 48 hours at 220 rpm. Following incubation, the 1 mL from each starter culture was transferred into a clean 1.5 microcentrifuge tube and centrifuged for five minutes at 13,200 rpm. The supernatant was decanted into a clean microcentrifuge tube and speed vacuumed for several hours at a low temperature. The pellets were resuspended in 95% acetonitrile: 5% of 1% acetic acid and then vortexed vigorously for 2 minutes. The samples were centrifuged at top speed for 5 minutes, decanted into clean centrifuge tube, and then syringe filtered using a 0.45 µm nylon filter into HPLC vials. The samples were analyzed using the Hewlett Packard Agilent 1100 series HPLC in which the 5 µM Prevail C-18 column. The chromatograms were collected using the LCQ Advantage Thermo Finnigan liquid chromatography coupled with ESI mass spectrometry detector under a gradient elution of 95:5 1% acetic acid:acetonitrile, with the acetonitrile increasing to 95% over a 45 minute linear gradient at 0.2 ml/minute flow rate.

To determine the difference in 48 hours incubation versus 72 hours incubation, the mutants were allowed to grow for another 24 hours. The extraction was followed as before using 5% of 1% acetic acid:95% acetonitrile as the solvent. After triturating the samples, each sample was applied to the HPLC-MS for analysis.

III.E.8 Characterization using *pksS* mutant, ethyl acetate, and LCMS

After receiving no concrete results, a new method was used to extract bacillaene from the cultures. The next extraction method used ethyl acetate as the resuspension

solvent during the trituration. The samples were prepared by making starter cultures containing 5 mL of LB media and 0.3 µg/mL of erythromycin that were inoculated with the mutants 2₁, 2₂, 3₁, and 3₂. 150 µL of the starter cultures were added to inoculate 50 mL Landy media cultures and were grown at 37°C for 48 hours at 220 rpm and then. Following incubation, the 1 mL from each starter culture was transferred into a clean 1.5 microcentrifuge tube and centrifuged for five minutes at 13,200 rpm. The supernatant was transferred into a microcentrifuge tube and speed vacuumed for several hours at a low temperature. In an attempt to resuspend the pellets, 1 mL of ethyl acetate was added to the pellet and the vortexed vigorously. After several minutes, the pellets were proved insoluble in ethyl acetate. The supernatant from the first extraction attempt was removed, transferred into a microcentrifuge tube, and recentrifuged. The supernatant was transferred to HPLC vial and applied to the HPLC-MS. The pellet that was determined to be insoluble in ethyl acetate, was then resuspended in a 95% acetonitrile:5% 1% acetic acid solution. The samples were centrifuged and the supernatant was transferred into HPLC vials. The samples were then applied to the HPLC-MS for analysis.

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